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Horizons don't get closer

But you extend them further

Every time you make some gains

So I keep on swimming in the waves of change

Spirit of the West

"Ships in Full Sail"

University of Alberta

Applications of Zwitterions in Capillary Electrophoresis

by

Mary Alice Woodland

A thesis submitted to the

Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry

Edmonton, Alberta

Fall 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled “Applications of Zwitterions in Capillary Electrophoresis” submitted by Mary Alice Woodland in partial fulfillment of the requirements for the degree of Master of Science.

Abstract

Capillary electrophoresis is a powerful tool for the separation of a wide variety of analytes from small inorganic anions to large biomolecules such as proteins. Many separations require additives to alter selectivity or prevent analyte adsorption to the capillary wall. Zwitterionic additives are advantageous since large concentrations can be used without contributing to the band broadening caused by Joule heating.

A novel method for altering the selectivity of inorganic anion separations is presented. Addition of the zwitterionic surfactant 3-(N,N-dimethyldodecylammonio)-propane sulfonate (DDAPS) to the background electrolyte modifies the migration order *via* electrostatic ion chromatography interactions. Variation of DDAPS concentration from 4 to 120 mM monotonically alters the selectivity from that based on electrophoretic mobility to that of electrostatic ion chromatography. This technique was applied to the determination of inorganic anions in seawater.

The adsorption of proteins to the silica capillary wall significantly reduces the efficiency of biological separations in capillary electrophoresis. High concentrations of zwitterions such as trimethylammoniumpropane sulfonate (Z1-Methyl) have been used in an effort to reduce protein-wall interactions. The effect of Z1-Methyl on protein behaviour in CE is investigated. A mechanism is proposed to explain the observed effects of Z1-Methyl on protein adsorption, mobility and electroosmotic flow.

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I am infinitely grateful to my fiancé Chris for his patience and support during the writing of this thesis. Thank you for being there through it all.

~ ~ ~

This thesis is dedicated to my fiancé and family.

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List of Symbols and Abbreviations

Symbol	Parameter
δ	dielectric increment, $\Delta\varepsilon/\Delta C$
ε	dielectric constant
ε_0	dielectric constant or permittivity of a vacuum, 8.854×10^{-12} $C^2/(N \cdot m^2)$
ε_W	dielectric constant of water, 78.48
η	viscosity
μ	mobility
μ_{app}	apparent mobility
μ_{eff}	effective mobility
μ^A_{eff}	effective mobility of anion
μ^C_{eff}	effective mobility of cation
μ^R_{eff}	effective mobility of a protein experiencing adsorption
μ_{ep}	electrophoretic mobility
μ_{EOF}	mobility of electroosmotic flow
σ^2	variance
ψ_o	potential at the capillary wall
ζ	zeta potential
ΔP	pressure difference
cmc	critical micelle concentration
d	inner diameter of capillary
k'	retention factor
pI	isoelectric point

pK_a	negative logarithm of the acid dissociation constant
q	charge
r	radius
t	migration time
t_0	elution time of unretained compound
t_A	migration time of anion
t_C	migration time of cation
t_{inj}	injection time
t_{migr}	time voltage is applied
t_{N1}	migration time of first neutral marker
t_{N2}	migration time of second neutral marker
t_r	retention time
$t_{ramp\text{-}down}$	time to ramp voltage from V_{appl} to zero
$t_{ramp\text{-}up}$	time to ramp voltage from zero to V_{appl}
v	velocity
$w_{0.1}$	peak width at one-tenth height
AU	absorbance units
BSA	bovine serum albumin
C	concentration
CAS U	cocoamidopropylhydroxyldimethylsulfobetaine
CE	capillary electrophoresis
C_{16}TAB	cetyltrimethylammonium bromide
C_{16}TAC	cetyltrimethylammonium chloride
D	molecular diffusion coefficient

DDAB	didodeyldimethylammonium bromide
DDAPS	3-(N,N-dimethyldodecylammonio)propane sulfonate
E	electric field
EOF	electroosmotic flow
F	Coulombic force
F	Faraday constant, 9.6487×10^4 C/mol
IC	ion chromatography
N_{bl}	baseline noise
L_d	capillary length to detector
L_T	total capillary length
LOD	limit of detection
M	molecular mass
MEKC	micellar electrokinetic chromatography
N	number of theoretical plates
ODS	octadecylsilane
PAGE	polyacrylamide gel electrophoresis
Q_P	protein charge
Q_W	wall charge
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
TR	transfer ratio
TTAB	tetradecyltrimethylammonium bromide
V_{appl}	applied voltage
V_s	partial specific volume

CHAPTER 1 INTRODUCTION

1.1 THE EVOLUTION OF CAPILLARY ELECTROPHORESIS

The advent of capillary electrophoresis on the separation scene resulted from the demands of two dramatically different areas of research. The rapid growth of the biotechnology industry required precise and high resolution methods to separate large molecules such as proteins. At the same time, worldwide concern for the declining state of the environment compelled the use of techniques which did not generate excessive waste.

In the 1960s, polyacrylamide gel electrophoresis (PAGE) was established as a rapid and inexpensive way to characterize proteins based on their molecular weights. This method was improved by adding a second dimension that separated proteins in a pH gradient based on their isoelectric points. [1] Presently, two-dimensional PAGE remains an effective technique for the separation of complex mixtures of thousands of proteins.

The early work of Hjertén [2] provides the first instance of electrophoresis in small diameter tubes of about 3 mm. The separation of a range of analytes from small inorganic anions to macromolecules such as proteins was demonstrated. However, no further progress in the field was made until the early 1980s. Improvements in detection, automation and fabrication of silica capillaries paved the way for the re-introduction of capillary electrophoresis (CE) by Jorgenson and Lukacs. [3] In the past twenty years, research in this field has grown dramatically. The scientific publications on CE numbered at about 2000 in the 1980s, while this sum ballooned to 3000 papers in the year 2000 alone. [4] At present, capillary electrophoresis enjoys a strong foothold in the area of separation science and the number of applications continues to grow.

1.2 CE INSTRUMENTATION

To a reasonable approximation, capillary electrophoresis (CE) separates analytes based on their charge-to-size ratio. This is effected by the application of a high electric field across a buffer-filled capillary. Figure 1.1 illustrates the major components of a CE system.

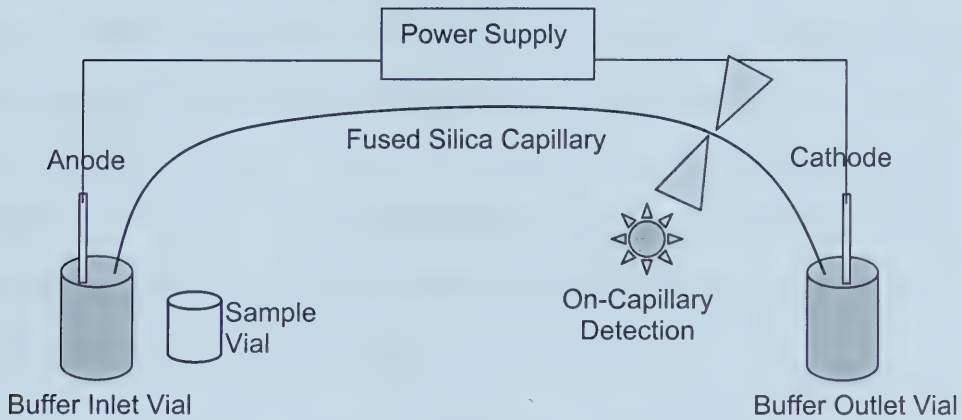

Figure 1.1 Schematic of a Capillary Electrophoresis Instrument

These components will be described in detail in the following sections. In brief, the two ends of a silica capillary are dipped into buffer reservoirs. In addition, two electrodes are dipped into these buffers and an electric field is applied. In the electrophoretic separation step, analytes move from the injector end to the detector at the opposite end. Detection is accomplished on-capillary and an electropherogram provides a plot of detector output as a function of migration time.

1.2.1 Sample Introduction

Injection of the sample into the capillary may be effected in several ways. The first method, electrokinetic injection, requires the application of voltage to the sample that the capillary is dipped in. The voltage causes the migration of the ionic components of the sample into the capillary. However, an obvious disadvantage of this technique is the discrimination between sample ions with different mobilities. The second injection method is hydrostatic injection which introduces sample by a siphoning action. Siphoning results from a difference in height between the buffer vial and sample vial on either end of the capillary. The last method, hydrodynamic injection, is the most commonly used technique in commercial instruments. A pressure difference is applied between the sample vial and the other end of the capillary to inject the sample. The amount of sample introduced is calculated from equation 1.1. [5]

$$V = \frac{\Delta P \pi d^4 t_{inj}}{128 \eta L_T} \qquad (1.1)$$

where V is the volume, ΔP is the pressure difference across the capillary, d is the inner diameter of the capillary, t_{inj} is the injection time, η is the buffer viscosity and L_T is the total capillary length. Typical injection conditions are 3 seconds at 0.5 psi (3.45 kPa). From eqn 1.1, this corresponds to 7 nL injected into a 27 cm long capillary with a 50 μm inner diameter. Hydrodynamic injection yields a relative standard deviation (RSD) in injection volume of 2 to 3% or as low as 1% if an internal standard is used. [5]

1.2.2 Separation

The separation step occurs in a fused silica capillary 20 to 100 cm in length. The external surface of the capillary is coated with polyimide to provide flexibility and strength. The capillary is filled with and dipped into a buffer solution in order to maintain a constant pH. To elicit a separation of analytes, a voltage of 5 to 30 kV is applied across the capillary. An inevitable result of using such high electric fields is the heating of the solution as current flows through it, an effect known as Joule heating. To prevent the undesirable consequences of Joule heating (discussed in Section 1.4.6), capillaries with an inner diameter of 20 to 100 μm and an outer diameter of 365 μm are used. This provides a high surface-to-volume ratio which efficiently dissipates the heat generated. In addition, the capillary is usually thermostated to ambient temperature by liquid or air cooling.

1.2.3 Detection

Detection is commonly performed on-capillary to avoid efficiency losses due to peak broadening outside the capillary. Optical detection methods are the most common, particularly UV absorbance and fluorescence. Since the polyimide coating is not UV transparent, it must be burned off at the detection window. This window is usually located about 7 to 10 cm from the terminal end of the capillary.

UV absorbance detection involves focusing the output of a UV light source, such as a deuterium arc lamp, into the detection window of the capillary. Optical filters or diffraction gratings are used to select the desired wavelength. A sample molecule possessing the appropriate chromophore absorbs a photon and is promoted to an

excited electronic state. This causes a decrease in the intensity of transmitted light reaching the photodetector. The absorbance signal is given by the Beer-Lambert law: [6]

$$Log\frac{I_0}{I} = A = \varepsilon bc \qquad (1.2)$$

where I_0 is the intensity of light incident upon the sample, I is the intensity of light emerging from the sample, A is the unitless absorbance, ε is the absorptivity of the sample, b is the pathlength and c is the concentration of sample. As stated in Section 1.2.2, it is necessary to use narrow capillaries (20 to 100 μm) in CE to minimize Joule heating. This limits the pathlength (b) for on-capillary detection to 20 to 100 μm. Under such conditions, the absorbance values will be low and one must try to detect a small difference between two relatively large signals. This is one of the fundamental limitations of absorbance detection. It becomes especially difficult if the sample concentration is low, as in most CE separations. Thus, typical detection limits for CE using direct UV absorbance are 10^{-6} M. [7]

1.2.3.1 Indirect Detection

In many analyses we are faced with the challenge of detecting analytes which do not possess a significant molar absorptivity. Indirect photometric detection addresses this problem by exploiting the lack of absorbance signal. A UV-absorbing species (called a probe), which has the same charge as the analyte, is added to the background buffer. This provides the detector with a constant background absorbance signal. The analyte, which presumably has little or no UV absorbance, displaces the probe ion

causing a quantifiable decrease in the background absorbance. Figure 1.2 illustrates the generation of this signal.

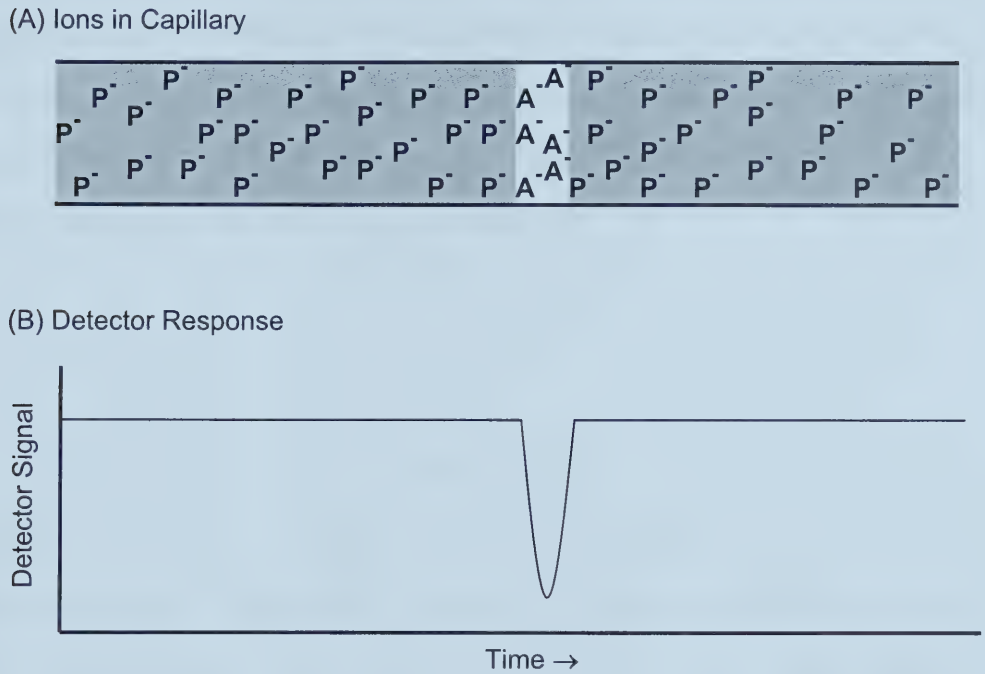

Figure 1.2 Schematic of Indirect Detection in CE. P^- represents the probe ion and A^- represents the analyte ion where $\varepsilon_{(P-)} > \varepsilon_{(A-)}$.

The result is a negative signal which is usually inverted to present the more visually pleasing positive peak. Since all ions exhibiting less absorbance than the probe should provide the same negative signal, indirect detection is a universal method of detection.

Although the concept appears simple, there are many factors to consider when choosing a suitable probe for indirect detection. First, the mobility of the probe must be closely matched to the mobility range of the analytes. If a large mismatch exists,

electrodispersion will occur (discussed in Section 1.4.4) and produce poor peak shapes. Analytes that migrate faster than the probe will give fronted peaks, while analytes that migrate slower than the probe will produce tailed peaks. Doble et al. [8, 9] provide the mobilities of some common probes and analytes which are useful in selecting an appropriate probe. A second requirement is that the probe should possess a high molar absorptivity in order to maximize the detection limits. The limit of detection (LOD) for a non-absorbing analyte using indirect absorbance is given by [9]

$$C_{LOD} = \frac{N_{bl}}{TR\varepsilon b} \qquad (1.3)$$

where N_{bl} is the baseline noise, TR is the transfer ratio (the number of moles of probe displaced by one mole of analyte), ε is the molar absorptivity of the probe and b is the detection pathlength. Finally, the presence of an additional co-ion from the buffer or a second probe may cause system peaks in the electropherogram. System peaks are baseline disturbances mimicking analyte peaks which may interfere with the peaks of interest. The preceding discussion provides a brief overview of indirect detection, leaving a more thorough description for literature reviews . [7-10]

1.3 PRINCIPLES OF CAPILLARY ELECTROPHORESIS

1.3.1 Electrophoretic Mobility

The application of an electric field across a solution will cause ions to move with velocity, v, according to the equation

$$v = \mu_{ep} E \qquad (1.4)$$

where μ_{ep} is the electrophoretic mobility of the analyte and E is the electric field (the applied voltage per length of capillary). Cations migrate towards the negative electrode or cathode, while anions migrate towards the positive electrode or anode. The electrophoretic mobility is a complex property of the charge and size of the ion, and has been the subject of much research in our laboratory. [11-14] However the essence of the charge-to-size dependence of mobility is given by the Hückel equation:

$$\mu = \frac{q}{6\pi\eta r} \tag{1.5}$$

where q is the analyte charge, η is the buffer viscosity and r is the Stokes' radius of the particle. Thus, a small, highly charged analyte will have a high mobility, whereas a large, minimally charged species will have a low mobility. Hence, separation in CE is primarily governed by the charge-to-size ratio of the analytes. The mass of the particle, M, is related to its size or Stokes' radius, r, by [15]

$$M = \frac{4}{3}\pi r^3 V_s \tag{1.6}$$

where V_s is the partial specific volume of the solute. As expected, analytes with a large molecular weight such as proteins will have a larger Stokes' radius and migrate slower than a low molecular weight ion such as bromide.

1.3.2 Electroosmotic Flow

Electroosmotic flow is the bulk flow of solution in the capillary and is a consequence of the surface charge on the interior capillary wall. Silanol (SiOH) groups on the capillary wall deprotonate to give the anionic form (SiO^-). The negatively charged

silanol groups attract cationic species from the buffer to maintain charge balance. The buildup of charge at the wall forms a double layer as shown in Figure 1.3.

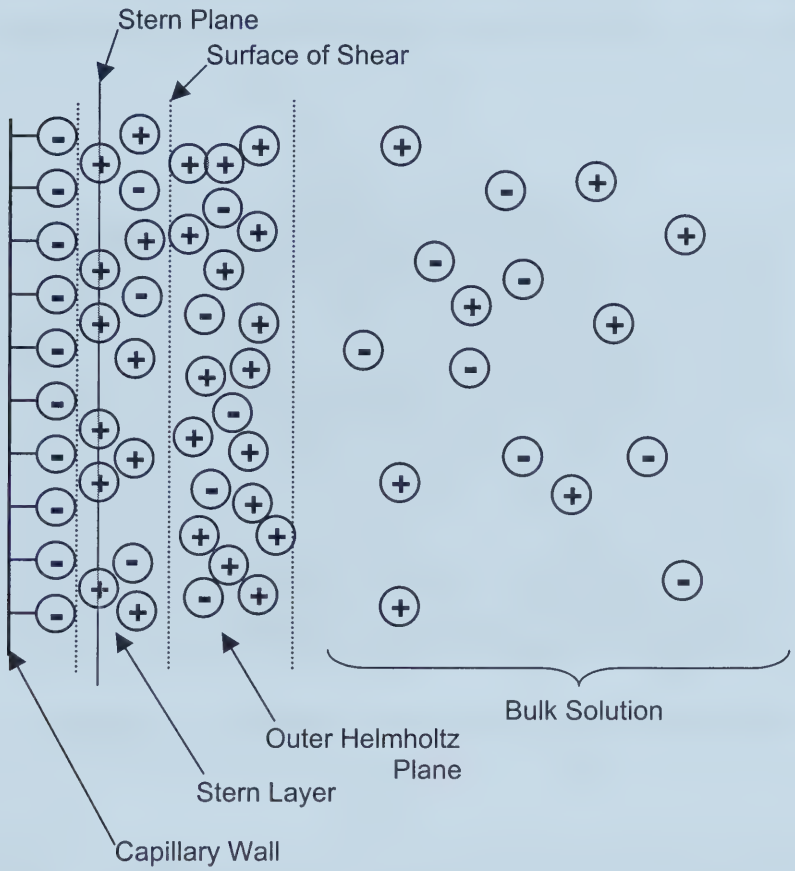

Figure 1.3 Schematic of the Double Layer at the Capillary Wall

Those ions intimately associated with the silanols are static and compose the Inner Helmholtz or Stern Layer. Bordering this fixed layer of cations is the Outer Helmholtz Plane, a more diffuse layer of cations which are movable. [16] The application of an electric field initiates the migration of cations in the Outer Helmholtz Layer towards the

cathode. These cations also drag their solvation shells with them. Since the water molecules in these shells form a strong hydrogen bond network with the water molecules of the bulk solution, the entire solution is pulled towards the negative electrode. Bulk flow towards the cathode is termed normal electroosmotic flow (EOF) and is illustrated in Figure 1.4.

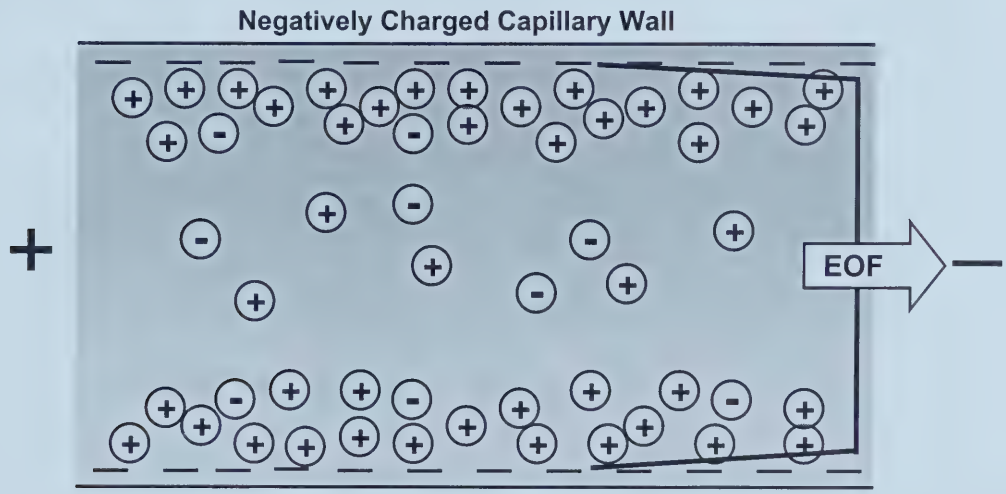

Figure 1.4 Generation of the Electoosmotic Flow (Thickness of diffuse region is exaggerated for clarity).

Generally, the EOF mobility is greater in magnitude than the electrophoretic mobility of the analytes. For instance, a typical EOF at neutral pH is 5 x 10^{-4} cm^2/Vs to 7 x 10^{-4} cm^2/Vs. [17] Common anions such as acetate and benzoate have electrophoretic mobilities of 4.24 x 10^{-4} cm^2/Vs and 3.36 x 10^{-4} cm^2/Vs, respectively. [18] For the most part, all species, including negatively charged and neutral compounds, are dragged towards the cathode by the EOF. Chapter 2 will discuss an exception of this rule since small inorganic anions are faster than the EOF and have a net mobility towards the anode.

A unique and beneficial characteristic of the EOF is its flat piston-like flow profile. No pressure drop exists since the flow is uniformly generated down the entire length of the capillary wall. In contrast, the flow in high pressure liquid chromatography (HPLC) is generated with an external pump. It produces a laminar or parabolic flow due to the pressure drop in the column. Figure 1.5 illustrates the peak shapes resulting from these flow profiles.

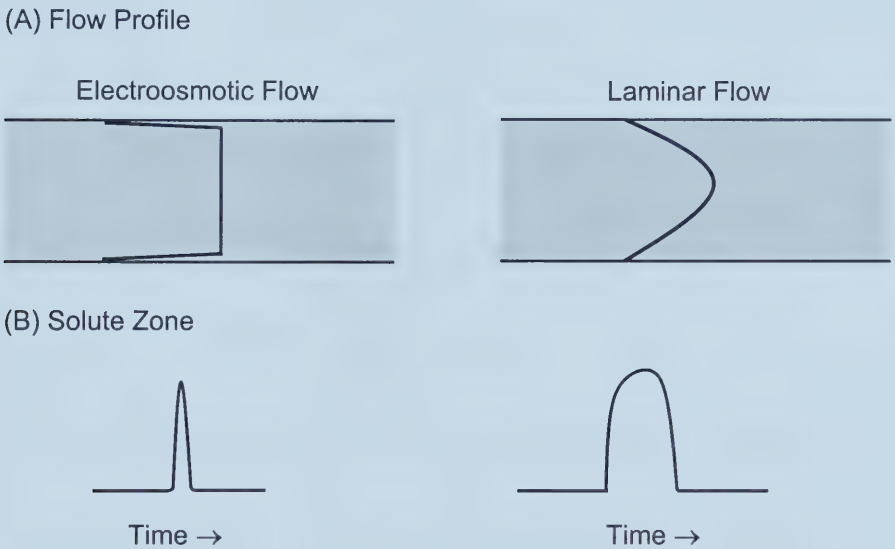

Figure 1.5 Effect of Flow Type on Peak Shape (A) Flow Profile (B) Resulting Analyte Peak Shape

Laminar flow contributes to the dispersion of the solute zone resulting in a much broader peak than that produced by the EOF.

At the capillary wall, the buildup of counter-ions creates a large potential difference, ψ_0. This potential difference decreases linearly to the Stern layer and then,

exponentially as the distance from the wall increases. Figure 1.6 traces the potential curve from the wall out to the bulk solution.

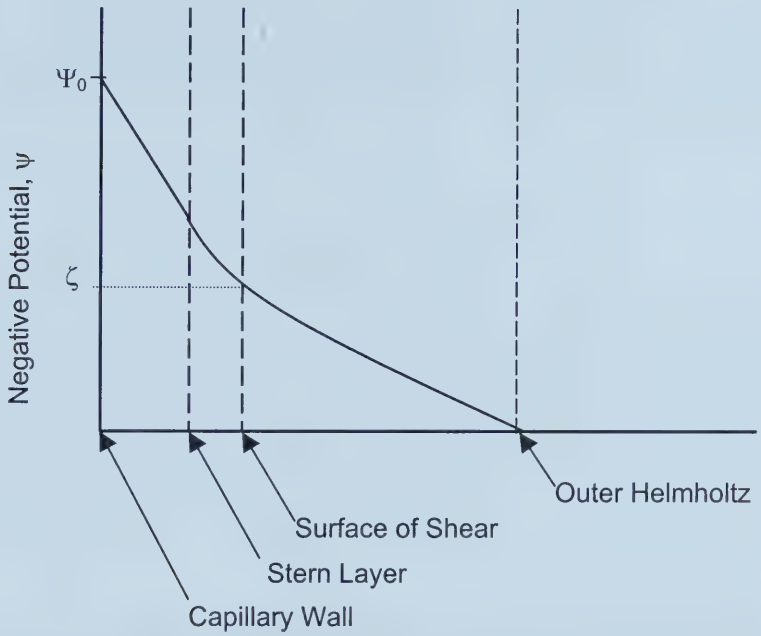

Figure 1.6 Potential as a Function of Distance from Capillary Wall

The surface of shear is defined at some distance from the wall and describes the point outside of which the associated cations are mobile. The EOF mobility is given by the von Smoluchowski equation, [16]

$$\mu_{EOF} = -\frac{\zeta\varepsilon}{\eta} \qquad (1.7)$$

where ζ is the zeta potential, ε is the dielectric constant of the solution and η is the viscosity of the solution. The zeta potential, ζ, is defined as the potential at the surface

of shear and is dependent upon the number of charges at the capillary wall. Since the latter is dictated by the number of deprotonated silanols on the wall, the EOF will vary with the pH of the buffer. The pK_a of the surface silanol groups is estimated to be 5.3. [19] If more silanols are in the anionic form, the zeta potential will be larger and as a result, the EOF is elevated.

1.3.3 Effect of EOF on Analyte Mobility

In most cases, the EOF moves all analytes, regardless of charge, in the same direction. Normal EOF from the anode to cathode moves the cations, neutral species and then, anions past the detector. The charge-to-size ratio causes a separation between analytes of the same charge as shown in Figure 1.7.

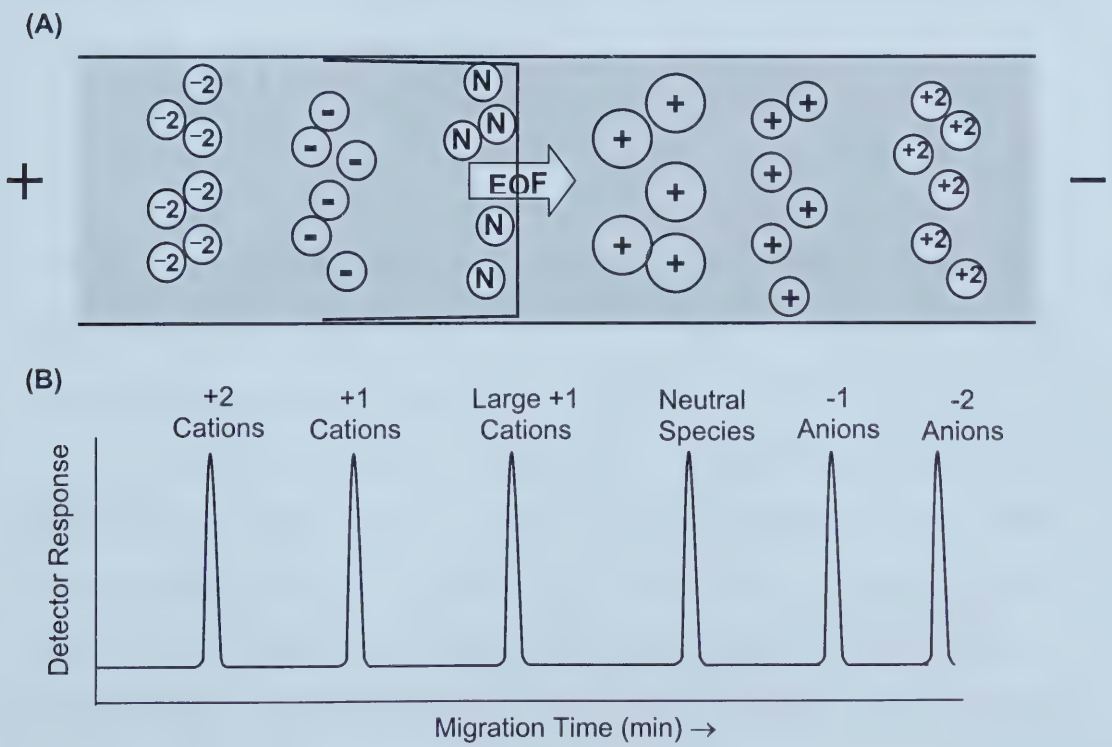

Figure 1.7 Mobility of Analytes in an Applied Field (A) Separation of Molecules in the Capillary (B) Resulting Electropherogram

Positive analytes migrate before the EOF, while negative analytes migrate after the EOF. In an electropherogram, the time required for an analyte to migrate past the detector is termed the migration time, *t*, and is used in eqn 1.8 to calculate the apparent mobility, μ_{app}.

$$\mu_{app} = \frac{L_d L_T}{t V_{appl}} \qquad (1.8)$$

where L_d is the capillary length to the detector, L_T is the total capillary length and V_{appl} is the applied voltage. As expected, analytes with a fast apparent mobility will reach the detector first and have a low migration time, while the opposite is true for slower ions.

The apparent mobility is the mobility measured in the presence of EOF. In an electrophoretic separation, the effective mobility or μ_{eff} is calculated by subtracting the EOF from the measured mobility as given by

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \qquad (1.9)$$

where μ_{app} is the apparent mobility and μ_{EOF} is the mobility of the EOF. The effective mobility is the intrinsic electrophoretic mobility of the analyte. It is caused solely by the action of the electric field and is determined by the Hückel eqn (1.5).

A measure of the magnitude of the EOF is accomplished by recording the migration time of a neutral marker. An uncharged analyte possesses no intrinsic mobility and moves solely with the EOF. Equation 1.8 can also be used to calculate the mobility of the EOF or μ_{EOF} where *t* is the migration time of the neutral marker. Some commonly used EOF markers include dimethyl formamide, [15] mesityl oxide, [20, 21] benzyl alcohol, [22] and acetone. [23]

1.4 SOURCES OF BAND BROADENING

There are a number of contributors to the peak broadening of a sample zone as it travels through the capillary. The extent of dispersion dictates the peak width which directly affects the efficiency and resolution of a separation. Peak broadening is described by the overall variance, σ^2_{total}, which is the sum of all sources of variance given by [24]

$$\sigma^2_{total} = \sigma^2_{extra} + \sigma^2_{coil} + \sigma^2_{diff} + \sigma^2_{disp} + \sigma^2_{ads} + \sigma^2_{Joule} \qquad (1.10)$$

where σ^2_{extra}, σ^2_{coil}, σ^2_{diff}, σ^2_{disp}, σ^2_{ads}, and σ^2_{Joule} are, respectively, the variances contributed by extracolumn effects, capillary coiling, longitudinal diffusion, electrodispersion, adsorption of solute onto the capillary wall and Joule heating. The validity of adding these variances is based on the assumption that these processes act independently of each other. Each of these sources will be discussed in the following sections.

1.4.1 Extracolumn Effects

Extracolumn sources of band broadening encompass those processes which do not occur during the separation in the capillary. These effects include variance contributions from the injection and detection of analytes.

Band broadening is present as early as the sample injection phase since the sample is introduced as a cylindrical plug, not as a thin disk. Sternberg [25] expressed the variance due to sample injection, σ^2_{inj}, as

$$\sigma^2_{inj} = \frac{l^2_{inj}}{12} \qquad (1.11)$$

where l^2_{inj} is the length of the injected sample plug. Sample injection contributes to band broadening when the ratio of sample length to capillary length is too large. [26, 27] In addition, eqn 1.11 assumes the injection of a rectangular plug which is not the case for hydrodynamic injection. Pressurized injection introduces the sample by laminar flow resulting in a parabolic sample profile. This sample shape further complicates the variance caused by sample injection.

Band broadening arising from the detector results from detector rise time, detection window length and data acquisition rate. A slow detector rise time is useful in smoothing out baseline noise, but too large a time constant can contribute to peak broadening. A large decrease in efficiency is observed with the use of slow time constants. [28] Next, the detector window length contributes to band broadening. The output of the detector is a signal which is averaged over the finite volume in contact with the detector window. The resulting variance is found by using the detection window length in place of sample plug length in eqn 1.11. [29] Last, the data acquisition rate or frequency with which data points are collected can cause variance. This rate should be as high as possible to ensure the peak is well defined.

1.4.2 Capillary Coiling

Many commercial instruments encase the capillary inside a cartridge to allow easy insertion into the instrument and to allow liquid thermostating. Longer capillary lengths are accommodated by wrapping the capillary around a wheel as many times as required. In coiled capillaries, the solute at the outer circumference travels a greater distance than the solute in the inner circumference. This discrepancy results in a spacial variance in migration time. This source of band broadening is cause for concern only for

small coil radii such as on microchips or in cases where the compensating act of diffusion is not present (i.e. large molecules). [24]

1.4.3 Longitudinal Diffusion

Longitudinal diffusion is the axial diffusion of the solute from the injection plug to the bulk solution caused by a concentration gradient. The variance resulting from diffusion, σ^2_{diff}, is [15]

$$\sigma^2_{diff} = 2Dt \qquad (1.12)$$

where D is the diffusion constant of the solute and t is the migration time of the solute. Thus, large molecules such as proteins will suffer less longitudinal diffusion than small molecules since the latter have higher diffusion constants. Also, analytes with a low electrophoretic mobility spend more time in the capillary and experience more diffusion. This source of variance can be minimized by decreasing the migration time of the analyte. Use of shorter capillaries and higher voltages will help accomplish this as shown in eqn 1.8. However, in many separations, generation of short migration times is at the expense of loss in resolution. Therefore, longitudinal diffusion remains to be the fundamental limitation of efficiency in CE. [27] The theoretical efficiency, N, achieved if the separation is limited only by longitudinal diffusion is given by [15]

$$N = \frac{L_d^2}{\sigma^2_{tot}} = \frac{L_d^2}{2Dt} \qquad (1.13)$$

where σ^2_{tot} is the total variance, L_d is the capillary length to the detector, D is the diffusion constant of the solute and t is the migration time of the analyte.

1.4.4 Electrodispersion

Electrodispersion is caused by variations in local field strength due to mismatched sample and buffer conductivities. Mobility is a function of conductivity according to the equation [30]

$$\mu = \frac{\lambda_{equiv}}{F} \qquad (1.14)$$

where λ_{equiv} is the limiting ionic equivalent conductance and F is the Faraday constant. Therefore, electrodispersion arises from a difference in mobility between the sample and the buffer.

When the solute zone has a lower mobility than the running buffer, a peak with a sharp leading edge and diffuse tailing edge will result. This is illustrated in Figure 1.8 shown on the following page. The solute zone has a lower conductivity and thus, higher electric field than the running buffer. Sample ions that diffuse into the buffer in the direction of migration encounter a lower field strength. Their mobility is slowed and the following sample ions are able to overtake them. In this way, a stacking effect at the front of the sample plug causes a sharp front zone. Ions diffusing from the sample zone into the following electrolyte zone will also experience a lower electric field and be slowed. However, due to their position behind the solute zone, they will lag behind causing a diffuse trailing edge. The sharp edge will reach the detector before the diffuse edge, giving the electropherogram shown in Figure 1.8.C.

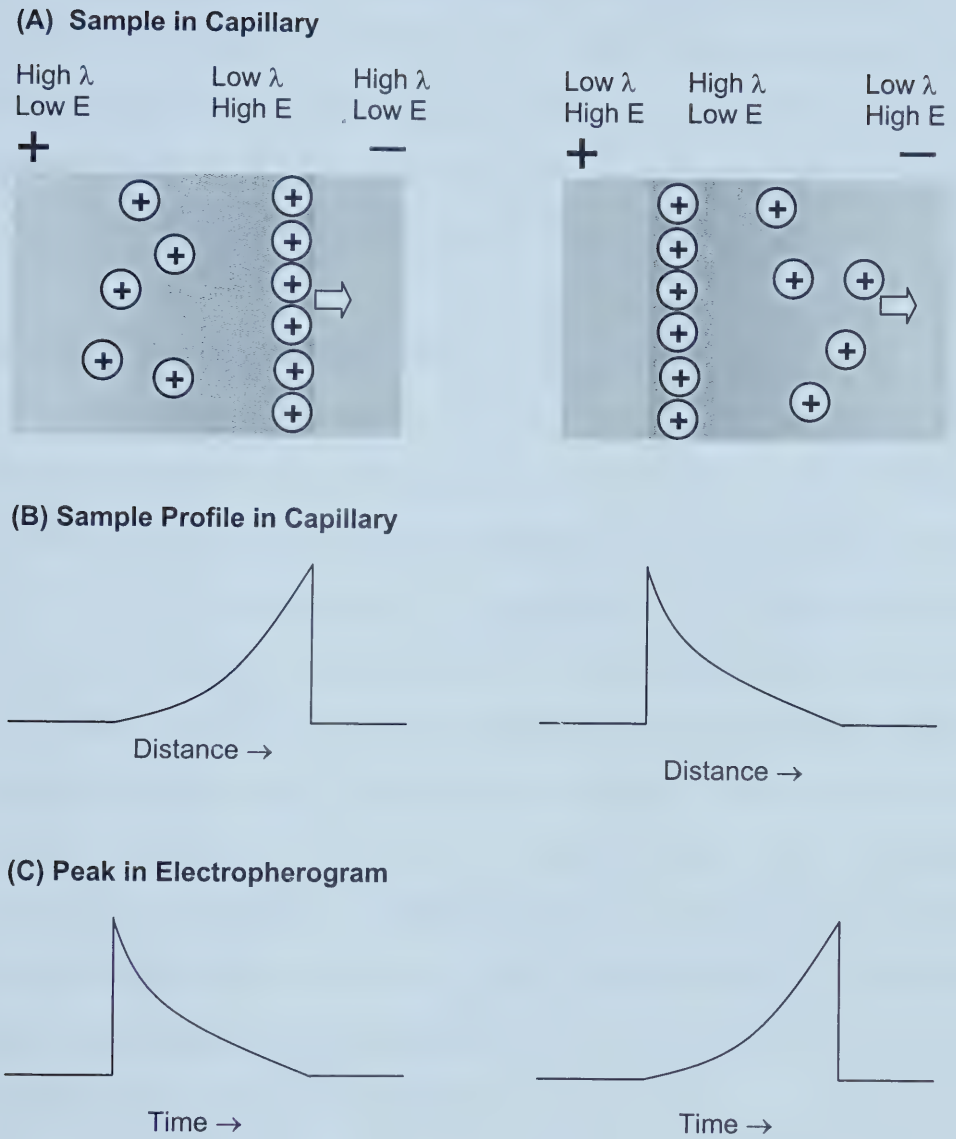

Figure 1.8 Band Broadening due to Electrodispersion. λ is conductivity and E is electric field strength

Conversely, a solute zone with a higher conductivity than the buffer zone will yield electrophoretic peaks with a diffuse front edge and sharp tail edge. In the case where sample and buffer conductivity are matched, no peak distortion will occur and Gaussian peaks result. This conductivity match is highly desirable when performing indirect detection, as previously discussed in Section 1.2.3.1.

1.4.5 Solute-Capillary Wall Adsorption

Based on longitudinal diffusion (Section 1.4.3) one would assume that efficiency would be greatest for high molecular weight analytes such as proteins. However, band broadening in the separation of proteins has posed a significant obstacle for the effective use of CE in biological applications. Interaction between the sample and the capillary wall causes a significant decrease in efficiency. A positively charged solute such as a protein below its pI is electrostatically attracted to the negatively charged capillary wall and may adsorb to the wall. When the analyte desorbs, it will elute at a later migration time. This results in a large variance in migration times as well as tailed peaks. McManigill and Swedberg [31] have studied the effect of reversible protein adsorption on efficiency and discovered that retention factors as low as 0.001 can adversely affect peak shape and efficiency. The methods that have been used to decrease protein-wall interactions will be discussed in Chapter 3.

1.4.6 Joule Heating

Joule heat is the increase in temperature caused by the passage of electrical current in the capillary. Band broadening does not result from the *absolute* increase in temperature, but it is induced by a temperature *gradient* across the capillary. The high

surface-to-volume ratio of the capillary dissipates heat efficiently at the wall. However, the heat at the center of the capillary is not as effectively dissipated causing a higher temperature. The result is a parabolic temperature profile as shown in Figure 1.9.

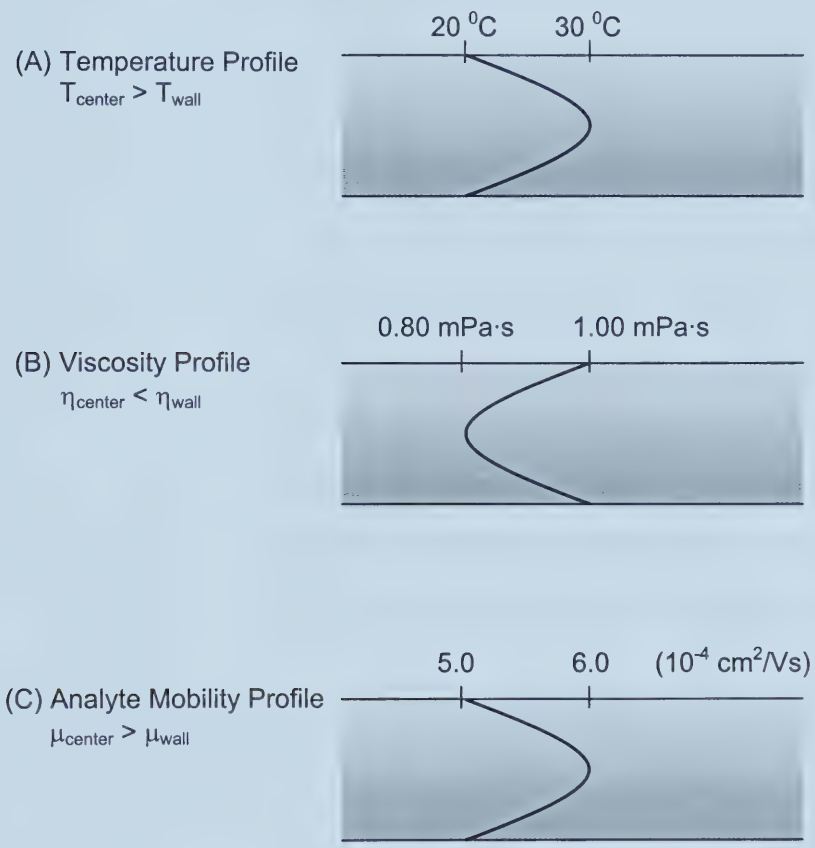

Figure 1.9 Effect of Joule Heating on (A) Temperature of the Capillary (B) Viscosity of the Buffer (C) Mobility of the Analyte

Since viscosity is inversely proportional to temperature, the viscosity will be decreased at the center of the capillary as much as 2% per ^{0}C. [32] The electrophoretic mobility is inversely related to viscosity according to the Hückel equation (eqn 1.5). This produces

a parabolic solute profile which contributes significantly to zone broadening. The variance caused by dispersion in the curved velocity profile is given by [15]

$$\sigma^2_{Joule} = \frac{r^6 E^4 C_b^2 B^2 \lambda^2 t}{1536 D k_b^2 T^4} \qquad (1.15)$$

where r is the inner radius of the capillary, E is the applied electric field, C_b is the concentration of buffer, B is a buffer-related viscosity constant, λ is the equivalent conductance of the electrolyte solution, t is the migration time of the analyte, D is the diffusion coefficient of the analyte, k_b is the thermal conductivity of the buffer and T is the absolute temperature.

The strong dependence of the variance on both the radius of the capillary and the electric field explains the motivation for performing high voltage CE separations in narrow bore capillaries. The inverse dependence on diffusion constant indicates that diffusion of solute from one part of the profile to another will diminish the parabolic mobility profile in Figure 1.9(C).

The presence of Joule heating can be detected by measuring the current, I, as a function of the applied voltage, V. This data should be linear according to Ohm's law,

$$I = \left(\frac{1}{R}\right) V \qquad (1.16)$$

where R is the resistance of the solution. At the point where an upward curvature in the plot is detected, the Joule heat generated is too large for the system to dissipate. Increases in current are observed since an increase in temperature decreases the

resistance of the solution. Figure 1.10 provides an example of an Ohm's law plot with deviations from linearity at high voltages.

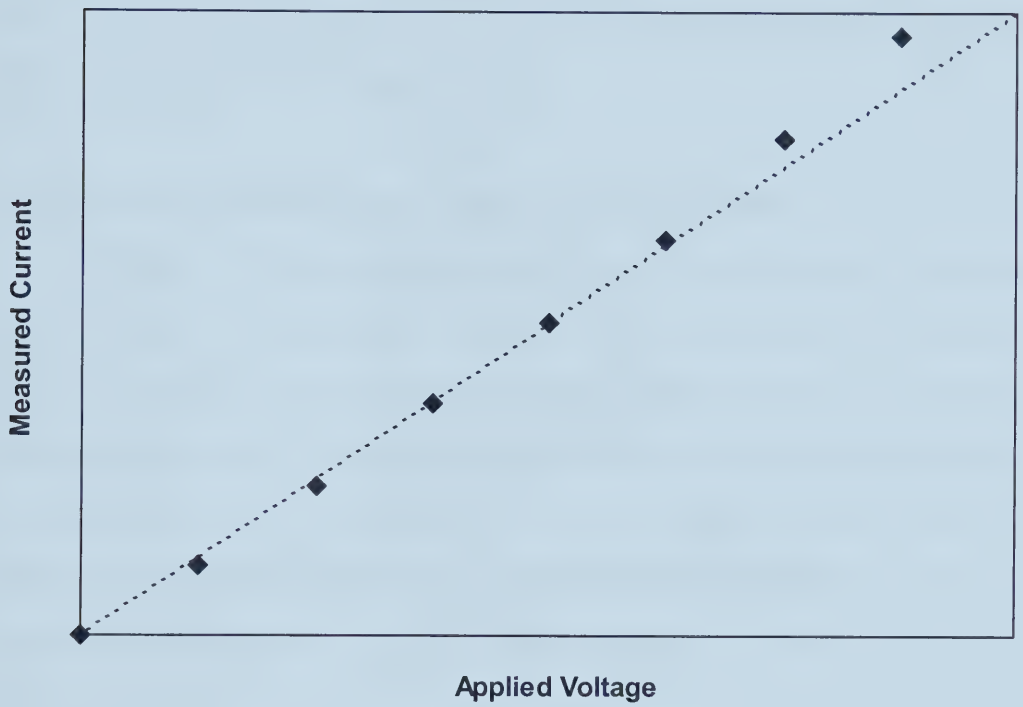

Figure 1.10 Ohm's Law Plot ◆ represents experimental data points and ----- represents the expected Ohm's law trend

Higher electric fields are desired for rapid separations but this must be balanced by the need to decrease Joule heating. Ohm's plots were performed for all buffers used herein, but are not shown.

Several alternate methods to reduce Joule heating are available. First, liquid thermostating of the capillary provides more efficient heat dissipation than air cooling. Second, decreasing the capillary radius will dramatically decrease the thermal gradient according to eqn 1.15. However, it also causes a simultaneous reduction in detection

sensitivity (Section 1.2.3). Third, a lower concentration of buffer may be used, but buffering capacity will suffer. Last, buffers with low conductivity will produce less current and Joule heat. In view of this, zwitterionic buffers or additives are ideal since their net charge and thus, conductivity, is zero. This thesis investigates the use of zwitterionic additives in capillary electrophoresis.

1.5 USE OF ZWITTERIONS IN CAPILLARY ELECTROPHORESIS

Zwitterions are electrically neutral compounds which possess both a positive and a negative charge. Provided both charged groups are completely, or at least equally, ionized, the net charge is zero and a true zwitterion has no conductivity. This property makes zwitterions an ideal additive in capillary electrophoresis which is plagued by Joule heating. Zwitterions are employed in CE in a number of roles. They are used as low conductivity buffers, as additives to alter EOF, as additives to modify the selectivity of the separation and as additives to prevent protein adsorption. The latter two roles will be the focus of this thesis.

1.5.1 Zwitterionic Buffers

In order for zwitterions to have sufficient buffering capacity, the absolute value of the difference between the pI and both pK_a values of the zwitterionic charged groups (or ½ ΔpK_a) should be less than one pH unit. [33, 34] Hjertén et al. [35] have designed zwitterionic buffers consisting of acidic and basic groups with the same pK_a value. These derivatized polyoxyethylene buffers allow the use of extremely high electric fields without inducing Joule heating. In fact, four benzoic acid derivatives were baseline separated in under 25 seconds with the use of 2000 V/cm! In a similar vein, Haddad

and coworkers [36] demonstrated the use of a novel polyethyleneimine isoelectric buffer for CE separations. Twenty anions were separated and the use of indirect photometric detection provided LOD from 0.2 to 1.6 μM. Such detection limits were possible because zwitterionic buffers do not interfere with indirect detection. That is, neutral species do no competitively displace the UV-absorbing probe ion. Furthermore, no system peaks (see Section 1.2.3.1) were observed in a 35 minute electrophoretic run. Haddad's group have also demonstrated that lysine and glutamic acid are effective zwitterionic buffers at their isoelectric points (pI). [37] Our group has used glutamic acid at its pI (pH 3.22) as a buffer for the indirect UV determination of chemical warfare degradation products. [38] The excellent migration time reproducibility (0.2 % RSD) obtained was a 10-fold improvement over previous studies with comparable sensitivity. Finally, zwitterionic buffers are also advantageous in CE with electrochemical detection due to the decreased current at the electrode. In this case, detection limits of 10 nM were attained in an amino acid separation. [39]

1.5.2 Alteration of Electroosmotic Flow using Zwitterionic Surfactants

Surfactants consist of a hydrophobic tail such as a long carbon chain and a hydrophilic head group which is usually charged. At concentrations above their critical micelle concentration (cmc), surfactants form micelles, that is, aggregates of surfactant monomers. [16] Zwitterionic surfactants consist of a hydrophobic tail and a hydrophilic head group which has both positive and negative functionalities. These surfactants may be added to the buffer to alter the magnitude or direction of the electroosmotic flow (EOF). Previous work in our group has demonstrated that zwitterionic surfactants such as CAS U (cocoamidopropylhydroxyldimethylsulfobetaine) suppress the EOF by up to

90%. [40] The onset of EOF suppression is correlated very well with the cmc of the surfactant. Thus, adsorption of zwitterionic micelles to the silanols of the capillary shields the wall charge and a subsequent decrease in EOF results. Yeung and Lucy [41] later added the zwitterionic surfactant, CAS U, to the commonly used cationic surfactant, C_{16}TAB (cetyltrimethylammonium bromide), to allow the baseline separation of [^{14}N]- and [^{15}N]aniline. C_{16}TAB reversed the EOF while CAS U suppressed its magnitude. Similar EOF control by mixed zwitterionic and cationic fluorosurfactants was employed for the simultaneous separation of cationic and anionic proteins. [42] Durkin and Foley [43] utilized sulfobetaine surfactants to suppress the EOF in their method of dual-opposite injection CE. In this technique, zero EOF is required for unbiased separation of both positively and negatively charged species. Cations and anions introduced at the anode and cathode, respectively, migrate with their electrophoretic mobility towards the detector located in the center of the capillary.

1.5.3 Alteration of Selectivity in Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) was first introduced by Terabe and coworkers in 1984. [44, 45] In this technique, surfactants are used above their cmc to form micelles with a hydrophobic core and a hydrophilic surface. Charged micelles migrate towards the corresponding oppositely charged electrode. However, their high mass slows their mobility and they are ultimately carried by the EOF. Solutes partition in and out of the micelle based on their hydrophobicity allowing the separation of neutral analytes. Very hydrophobic analytes spend most of their time in the micelle and elute later than hydrophilic analytes which spend more time in the bulk solution phase.

Generally, anionic surfactants such as SDS (sodium dodecyl sulfate) or cationic surfactants such as C_{16}TAB are used in MEKC. [20] However, there are numerous examples of zwitterionic surfactants being used as the pseudo-stationary phase in MEKC. [46-50] The use of DDAPS (3-(N,N-dimethyldodecylammonio)propane sulfonate), a zwitterionic surfactant, enabled separation of peptides which differed by only one out of 23 amino acids. [48] Iso and Okada [47] controlled the extent of ionic solute interaction with the zwitterionic micelle by changing its zeta potential. The separation of insulin-like polypeptides was performed using DDAPS in MEKC. [50] In this work, Nashabeh et al. also demonstrated that zwitterionic surfactants are compatible with on-line CE-mass spectrometry. In capillaries where EOF has been suppressed, neutral zwitterionic micelles do not migrate into the mass spectrometer and thus, do not interfere with peak detection and identification.

Zwitterionic surfactants offer an alternate selectivity when combined with anionic or neutral surfactants in mixed micelle MEKC. [42, 43, 51-53] Selectivity enhancement is achieved without the increase in analysis time observed for organic modifiers [53] or an increase in conductivity. Kang et al. [51] demonstrated that a sulfobetaine zwitterionic surfactant offered more selectivity than a nonionic surfactant (Brij 35). In this manner, the separation of 50 components of bacitracin, a polypeptide antibiotic, was successful.

An alternative method to MEKC, which uses zwitterionic surfactants to provide selectivity changes in anions, will be discussed in Chapter 2.

1.5.4 Prevention of Protein Adsorption

Zwitterionic surfactants have been shown to efficiently coat the capillary wall and reduce protein-wall interactions. The simultaneous separation of anionic and cationic

proteins using a sulfobetaine zwitterionic surfactant yielded efficiencies approaching a million plates/m. [54] Strege and Lagu [55] used the zwitterionic surfactant, DDAPS, to coat the wall of a poly(acrylamide)-derivatized capillary. The incorporation of DDAPS vastly improved peak symmetry and resulted in a migration time reproducibility of 0.7% RSD. In other work, the coating of a zwitterionic surfactant on a C_8-derivatized capillary also produced efficient protein separations. [56]

A further advantage of zwitterionic additives is they are non-denaturing, that is, they do not perturb the secondary and tertiary structure of biopolymers such as proteins. [48, 49, 57]

Zwitterions (with no surfactant functionality i.e. lacking a hydrophobic tail) have also been used to decrease band broadening resulting from protein adsorption. This approach was first introduced by Bushey and Jorgenson in 1989. [23] Since their pioneering work, zwitterions have been employed to improve the efficiency of many biological separations. [58-65] Several mechanisms for their effectiveness have been proposed including ionic screening and competition for silanol ion exchange sites. The use of zwitterions to prevent protein-wall interactions will be discussed in detail in Chapter 3. An explanation for the observed reduction in band broadening of proteins will be presented.

1.6 OUTLINE OF THESIS

Zwitterions possess both positive and negative groups, but their net charge and conductivity is zero. As a result, large concentrations can be employed without inducing Joule heating. This makes them ideal additives in capillary electrophoresis. The preceding discussion outlined some of the applications in which zwitterions are used.

This thesis studies some of these applications. In Chapter 2, zwitterionic surfactants are used to alter the selectivity of inorganic anion separations. This method is applied to the determination of these anions in seawater. Chapter 3 probes the use of zwitterions in preventing the adsorption of proteins to the capillary wall. Particular attention will be paid to understanding the mechanism by which trimethylammonium-propane sulfonate (Z1-Methyl) reduces protein-wall interactions.

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CHAPTER 2: ALTERING SELECTIVITY USING ELECTROSTATIC CE ‡

2.1 INTRODUCTION

The analysis of small inorganic anions is important in a variety of fields such as food, clinical and biological sciences, as well as environmental and industrial applications. [1] Although traditionally performed using ion chromatography, [1] capillary electrophoresis (CE) offers more efficient and rapid separations of these analytes. [2] A wealth of papers have been published on methods to alter the selectivity in CE. [3-8] Even considering the vast amount of work done (reference 5 quotes 293 papers), the existing methods remain limited, especially for small inorganic anions.

Introduction of secondary equilibria is one of the most effective means of altering selectivity in CE. However complexation equilibria, such as those used with metal cations, [9-11] are generally not possible with anionic species. More commonly acid/base equilibria or ion exchange equilibria are used. It is well known that selectivity changes will occur when the pK_a of the analyte is near that of the buffer. Thus, acid dissociation equilibria offer an effective means of altering the mobility of weakly acid species such as phosphate, [12] borate, [2] carbonate [2, 13] and nitrite. [14, 15] Alas, this approach is of little use to vary the mobilities of inorganic species such as chloride, bromide, nitrate and iodide whose acids possess unattainable pK_a values. [16]

Alkylammonium surfactants such as tetradecyltrimethylammonium bromide (TTAB) are commonly added to the buffer to reverse the electroosmotic flow (EOF) for

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anionic separations. [2, 13] In addition to acting as flow modifiers, it was noted that the concentration of alkylammonium surfactant affects the migration times of some inorganic anions through ion pairing interactions. [2, 17, 18] Studies of inorganic anion migration in micellar electrokinetic capillary chromatography (MEKC) demonstrated that anion migration was retarded by ion association with free surfactant monomers as well as distribution into the cationic micelles. [17] It has also been suggested that the selectivity changes observed with the use of cationic surfactants result from ion exchange partitioning effects. [4, 19, 20]

The ability to introduce an ion exchange element to capillary electrophoresis separations is particularly powerful since these two techniques offer complementary selectivity. [21, 22] Ion exchange selectivity may be more directly introduced into CZE by addition of polycationic species such as are poly(1,1-dimethyl-3,5-dimethylenepiperidinium) (PDDPi), poly (1,1-dimethyl-3,5-dimethylenepyrrolidinium) (PDDPy) and poly(diallyldimethylammonium chloride) (PDDAC). [23, 24] The degree to which ion exchange retards anion mobility is directly related to the concentration of polyelectrolyte. Also, variation of the electrolyte concentration in the background buffer changes the degree to which the analyte interacts with the polymer and its resultant migration time. [24] These parameters allow the researcher to adjust the extent to which the separation occurs via electrophoretic or ion-exchange effects.

Recently, electrostatic ion chromatography (IC) was introduced for the chromatographic separation of inorganic ions. [25-33] Electrostatic IC uses an octadecylsilane (ODS) column coated with a zwitterionic surfactant containing both anionic and cationic functional groups. In this technique, equilibration of the background electrolyte with the zwitterion establishes a charged layer around the surfactant head

group. The charge and magnitude of this layer dictates the ease with which analyte ions can penetrate it. Once inside the charge barrier, anions are retained based on chaotropic interaction with the quaternary ammonium group according to the Hofmeister series. [34] As a result, the order of elution for electrostatic ion chromatography differs dramatically from that of classical ion exchange chromatography or capillary zone electrophoresis. [21, 29]

This study aims to combine the speed and efficiency of capillary electrophoresis with the entirely different selectivity offered by electrostatic ion chromatography. A zwitterionic surfactant is added directly to the electrophoretic buffer to alter the selectivity of inorganic anionic separations. The concentration of zwitterion used dictates the resulting migration order of the anions since it determines the extent of electrophoretic or chromatographic influence on the separation. A particular advantage of using zwitterionic agents is that high concentrations can be used without inducing Joule heating.

The use of zwitterionic surfactants to alter selectivity was applied to the determination of iodide, nitrate, nitrite and bromide in artificial seawater. This separation is conventionally done by ion chromatography [1] and is one of the more difficult matrices to deal with in CE. A major challenge in quantifying species in seawater is the interference of matrix components such as chloride which hinder the reliability of the analysis. In this work, we will use our ability to alter the migration times and order of the anions to remove the analyte peaks from the area affected by the matrix peaks.

2.2 EXPERIMENTAL

2.2.1 Apparatus

Experiments were performed using a Beckman P/ACE 2100 system (Beckman Instruments, Fullerton, CA) with UV absorbance detection. Data acquisition (5 Hz) and control was performed using P/ACE Station software for Windows 95 (Beckman) on a Pentium 120 MHz microcomputer.

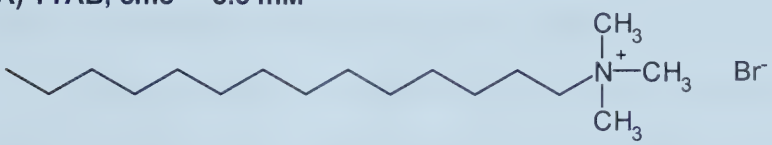
Untreated silica capillaries (Polymicro Technologies, Phoenix, AZ) with an inner diameter of 50 μm, outer diameter of 365 μm, and total length of 47 cm (40 cm to detector) were used. New capillaries were pre-treated with a high pressure (138 kPa) rinse using 0.1 M sodium hydroxide (BDH, Toronto, ON) for 5 min. Prior to each run, the capillary was rinsed at high pressure with 0.1 M sodium hydroxide for 1 min, water for 1 min and buffer for 2 min. In all experiments, the capillary was thermostated to 25°C using liquid coolant.

2.2.2 Reagents

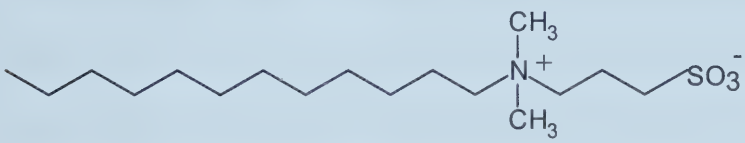
All solutions were prepared in Nanopure (18 MΩ) water (Barnstead, Chicago, IL). Buffers were prepared from reagent grade orthophosphoric acid (BDH) with sodium hydroxide (BDH) to adjust the pH, or potassium chromate (BDH) with phosphoric acid (BDH) to adjust the pH. The pH values were measured using a Corning digital pH meter model 445 (Corning, Acton, MA) calibrated immediately prior to use. 2 mM mesityl oxide (Aldrich) in water was used as the neutral EOF marker in direct detection studies. Previous research has demonstrated that mesityl oxide is an appropriate EOF marker in micellar media. [35, 36] Water was used as an EOF marker in experiments where indirect detection was used. The surfactants tetradecyltrimethylammonium bromide (TTAB)

(Sigma, St. Louis, MO), 3-(N,N-dimethyldodecylammonio)propane sulfonate (DDAPS) (Aldrich, Milwaukee, WI), 3-(N,N-dimethyloctylammonio)propane sulfonate (Zwittergent 3-08) (Calbiochem, San Diego, CA) and polyoxyethylene (23) dodecyl ether (Brij 35) (Aldrich) were used as received. Figure 2.1 provides the structures and critical micelle concentration (cmc) values of these surfactants.

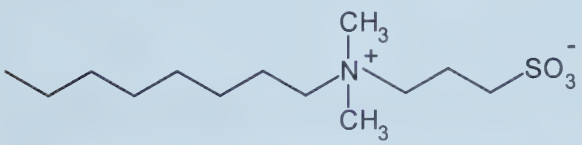
(A) TTAB, cmc = 3.5 mM [37]



(B) DDAPS, cmc = 2 to 4 mM [38]**, pK_a of SO_3^- group ≈ 1.5** [39]



(C) Zwittergent 3-08, cmc = 330 mM [38]**, pK_a of SO_3^- group ≈ 1.5** [39]



(D) Brij 35, cmc = 0.92 mM [38]

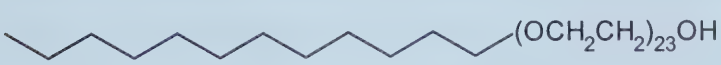


Figure 2.1 Structures of Surfactants Used (A) Cationic Surfactant, TTAB (B) Zwitterionic Surfactant, DDAPS (C) Zwitterionic Surfactant, Zwittergent 3-08 (D) Nonionic Surfactant, Brij 35. The quaternary-substituted ammonium group is charged over all pH values.

Surfactant solutions were made by adding the surfactant to the buffer solution prior to adjusting the pH and making up to volume.

Samples of 0.5 mM anion solutions in water were prepared from sodium nitrite (BDH), potassium nitrate (BDH), potassium bromide (Fisher Scientific, Fair Lawn, NJ), sodium fluoride (Fisher Scientific), sodium chloride (BDH), sodium sulfate (Fisher Scientific), potassium oxalate (Matheson, Norwood, OH), sodium iodide (BDH) and potassium thiocyanate (BDH) without further purification.

Artificial seawater was prepared by the method of Lyman and Fleming [40] with the exception that bromide ion was omitted and a 1 to 5 dilution was made prior to analysis. The final concentrations were as follows: 80.4 mM NaCl (BDH); 4.90 mM $MgCl_2$ (BDH); 5.52 mM Na_2SO_4 (BDH); 1.50 mM $CaCl_2$ (Anachemia, Rouses Point, NY); 1.78 mM KCl (BDH); 458 μM $NaHCO_3$ (BDH); 82.3 μM H_3BO_3 (BDH); 18.2 μM $SrCl_2$ (Fisher) and 15.0 μM NaF (Fisher). Standard dilutions of a stock solution of 5 mM NaI, KNO_3, KBr and $NaNO_2$ in artificial seawater were used to generate samples of the desired concentrations.

2.2.3 Effect of TTAB, a Cationic Surfactant, on Anion Mobility

The effect of the concentration of a cationic surfactant on selectivity was studied by adding 5 to 150 mM TTAB (Fig. 2.1A) to a 10 mM phosphate buffer at pH 7.2. A mixture of 0.5 mM $NaNO_2$, KNO_3, NaI and KSCN was hydrodynamically injected using low pressure (3.45 kPa) for 1.0 s. The applied potential was -20 kV and the detector rise time was 1.0 s. The direction of the EOF is reversed when using a cationic surfactant (i.e. from the cathode to the anode) so that the anions were separated in the co-EOF mode. Detection was near the anode using direct UV absorbance at 214 nm. Standard

additions of each analyte were performed at each concentration to determine the migration order. Duplicates of each run were performed.

The apparent mobility, μ_{app}, of the anions was determined from the migration time under constant voltage conditions using the following equation.

$$\mu_{app} = \frac{L_T L_d}{t V_{appl}} \qquad (2.1)$$

where L_T is the total capillary length, L_d is the effective capillary length to the detector, t is the migration time and V_{appl} is the applied voltage.

EOF was measured in the TTAB solutions by the conventional method of injecting a neutral marker, namely mesityl oxide, for 1 s using low pressure. A constant voltage of 20 kV was applied (from anode to cathode) for 7.5 min and the neutral compound is eluted from the capillary with the generated EOF. Direct absorbance detection was used at 254 nm. Equation 2.1 was applied to calculate the EOF mobility by inserting the migration time of the neutral marker.

Finally, the effective mobility of the analytes, μ_{eff}, corrected for EOF, was calculated using the equation

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \qquad (2.2)$$

where μ_{app} is the apparent mobility of the analyte and μ_{EOF} is the mobility of the EOF.

2.2.4 Effect of DDAPS, a Zwitterionic Surfactant, on Anion Mobility

The effect of the concentration of a zwitterionic surfactant on the selectivity was investigated by adding 3.75 to 120 mM DDAPS (Fig. 2.1B) to 5 mM chromate buffer at pH 8.0. A mixture of 0.5 mM $NaNO_2$, KNO_3, KBr, NaI, KSCN, NaF, NaCl, Na_2SO_4 and $K_2C_2O_4$ in water was separated. The sample was injected using low pressure (3.45 kPa) for 1.0 s. The applied potential was –20 kV and the detection rise time was 1.0 s. The EOF was from the anode to the cathode (normal EOF) so that anions were separated in the counter-EOF mode. Small inorganic anions have a higher absolute mobility than the electroosmotic mobility and are detected at the anode using indirect UV absorbance (254 nm). Standard additions of each analyte were performed at each surfactant concentration to determine the analyte migration order. The apparent mobility, μ_{app}, of the anions was determined using eqn 2.1, and the effective mobility of the analytes, μ_{eff}, corrected for EOF was calculated using eqn 2.2.

The electroosmotic flow generated in the presence of zwitterionic surfactants is slow. As a consequence, conventional measurement is impractical due to the length of time required to elute a neutral marker. Therefore, the EOF was measured in the DDAPS solutions using the three-peak injection method of Williams and Vigh. [41] In this procedure, the capillary was first rinsed and filled with the buffer. The EOF marker, water for indirect detection, was injected using low pressure (3.45 kPa) for 1.0 s. This band was pushed through the capillary using low pressure for 0.5 min. A second water sample was introduced as before and another low pressure push was applied for 0.5 min. A constant voltage of 10 kV was then applied from the anode to the cathode for 99 s. The position of the two neutral marker peaks was altered by the resultant EOF. A final water peak was injected and the bands were swept from the capillary by applying

low pressure for 15 min. Indirect absorbance detection was performed at 254 nm. The EOF was finally calculated using equation 2.3.

$$\mu_{EOF} = \frac{L_T L_d [(t_3 - t_2) - (t_2 - t_1)]}{V_{appl}(t_3 + t_{inj}/2)(t_{migr} - t_{ramp-up}/2 - t_{ramp-down}/2)} \qquad (2.3)$$

where t_1, t_2 and t_3 are the times required to push bands 1, 2 and 3 respectively past the detector. L_T is the total length of the capillary; L_d is the effective capillary length to the detector; V_{appl} is the applied voltage; t_{inj} is the injection time; t_{migr} is the time the voltage is applied and $t_{ramp-up}$ and $t_{ramp-down}$ are the times required to linearly change the applied potential between zero and V_{appl} (10.2 s for this study).

2.2.5 Effect of Zwittergent 3-08, a Zwitterion, on Anion Mobility

The effect of a zwitterionic surfactant below its cmc on anion mobility was determined by adding 5 to 150 mM Zwittergent 3-08 (Fig. 2.1C) to 10 mM phosphate buffer at pH 7.21. A mixture of 0.5 mM $NaNO_2$, KNO_3, NaI, KSCN and KBr was injected using low pressure (3.45 kPa) for 1.0 s. The applied potential was -20 kV and the detector rise time was 1.0 s. The EOF was from the anode to the cathode (normal EOF) so that the anions were separated in the counter-EOF mode. Detection was by the anode using direct UV absorbance at 214 nm. Standard additions of each analyte were performed at each additive concentration to determine the analyte migration order.

Since the zwitterionic surfactant is used below its cmc, no micelles are present and the EOF is not significantly suppressed. Thus, the EOF was measured in the Zwittergent 3-08 solutions by the conventional method of injecting a neutral marker, namely mesityl oxide, for 1 s using low pressure. A constant voltage of 20 kV was applied from the anode to the cathode. Direct absorbance detection near the cathode

was performed at 254 nm with a rise time of 1.0 s. Finally, the effective mobility of the anions was calculated using eqn 2.1 and eqn 2.2.

2.2.6 Effect of Brij 35, a Nonionic Surfactant, on Anion Mobility

The effect of a nonionic surfactant on anion mobility was determined by adding 10 to 75 mM Brij 35 (Fig. 2.1D) to 10 mM phosphate buffer at pH 7.21. A mixture of 0.5 mM $NaNO_2$, KNO_3, NaI, KSCN and KBr was injected using low pressure (3.45 kPa) for 1.0 s. The applied potential was -20 kV and the detector rise time was 1.0 s. The EOF was from the anode to the cathode (normal EOF) so that the anions were separated in the counter-EOF mode. Detection was at the anode using direct UV absorbance at 214 nm. Standard additions of each analyte were performed at each surfactant concentration to determine the analyte migration order.

The addition of nonionic surfactant to the buffer suppresses the EOF. Since conventional EOF measurement is impractical due to long run times, the three-peak method of Williams and Vigh [41] was employed. The method and experimental details are the same as in Section 2.2.4. Equations 2.2 and 2.3 were used to obtain the effective mobility of the anions.

2.2.7 Determination of Anions in Seawater

The separation of anions in seawater was accomplished. A mixture of 75 μM KBr, KNO_3, and $NaNO_2$ in 1:5 diluted seawater (Sec. 2.2.2) was injected using low pressure for 3 s and separated in 30 mM DDAPS 10 mM phosphate buffer at pH 8.0. Alternatively a mixture of 150 μM NaI, KNO_3, and $NaNO_2$ in 1:5 diluted seawater was injected using low pressure for 1 s and separated in 10 mM DDAPS 10 mM phosphate

buffer at pH 8.0. Both separations were done in counter-EOF mode using an applied voltage of -20 kV. Direct absorbance at 214 nm was used with a rise time of 1.0 s.

Limits of detection of the anions in seawater were determined using a procedure based on the U.S. Environmental Protection Agency methodology. [42] This approach determines the minimum amount of sample that can be reported to be greater than the background noise (blank run) with 95% confidence. Using the experimental conditions described in Section 2.2.4, a calibration curve was generated over the range 0.05 to 1.0 mM using dilutions of the 5 mM stock solution of the anions. Then, a 75 μM sample of $NaNO_2$, KNO_3 and KBr (about 5 to 10 times the estimated detection limit) in 1:5 diluted seawater was separated 9 times using 30 mM DDAPS in 10 mM phosphate at pH 8.0. The standard deviation for these replicate injections was determined. The detection limit is the standard deviation multiplied by the Student's *t* value. For n-1 or 8 degrees of freedom, the *t* value for the one-sided 95% confidence interval is 1.860. The same procedure was used to determine iodide detection limits using 10 mM DDAPS in 10 mM phosphate pH 8.0.

2.3 RESULTS AND DISCUSSION

2.3.1 Effect of TTAB Concentration on Anion Mobility

TTAB is a cationic surfactant commonly used to reverse the EOF in separations of inorganic and small organic anions. [2, 13] Selectivity changes have been reported with changes in the TTAB concentration. Jones and Jandik [2] described a change in migration order for bromide, chloride, nitrite and sulfate with the use of up to 5 mM TTAB in chromate buffer. Jimidar and Massart [20] also showed that bromide crosses over chloride when using up to 1 mM TTAB. These anion selectivity changes with increasing

TTAB concentration result from the electrostatic ion pairing between the negatively charged analyte and positively charged surfactant monomers and/or micelles in bulk solution. [17, 18, 43]

We wished to investigate whether more dramatic selectivity changes could be achieved by using higher concentrations of TTAB. However, a number of difficulties were experienced. First, TTAB is insoluble in chromate, the most common probe for inorganic anions, at pH less than 8.0. [12] This makes TTAB unsuitable for indirect detection at low pH. Second, severe baseline disturbances were observed when high concentrations of TTAB (> 5 mM) were added to the chromate buffer (pH 8.0). Third, high concentrations of TTAB significantly increase the current, as shown in Figure 2.2.

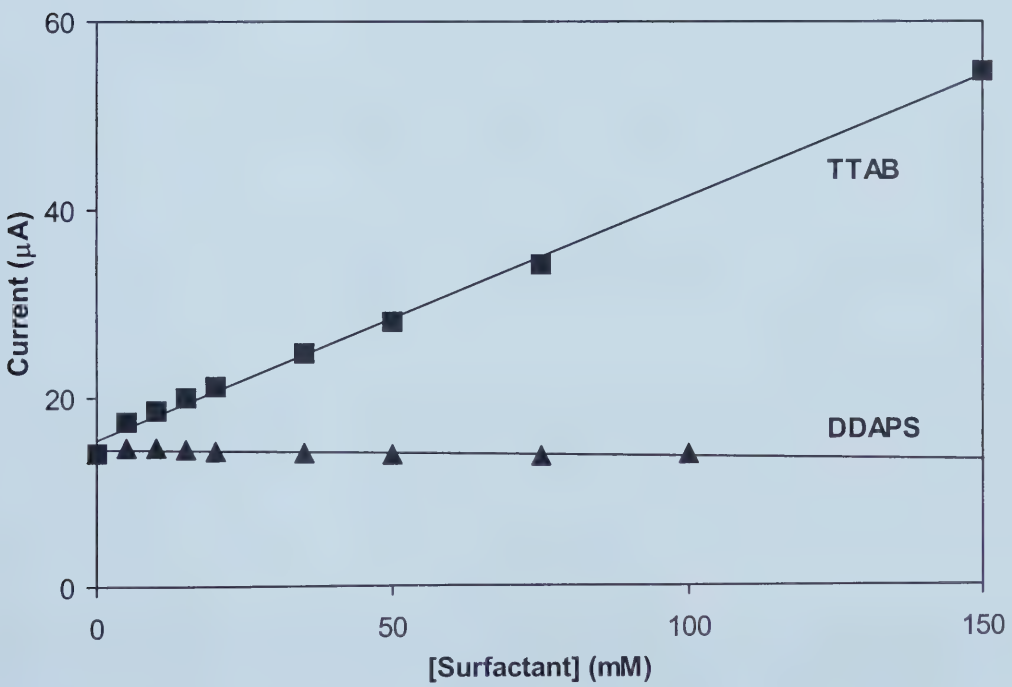


Figure 2.2 Effect of Type and Concentration of Surfactant on Current. Symbols are experimentally determined currents and lines are the best fit linear regression through the data. Refer to Sections 2.2.3 and 2.2.4 for experimental conditions.

The current produced by a zwitterionic surfactant, DDAPS, is shown for comparison. The high currents generated in TTAB solutions are undesirable as they will cause Joule heating and band broadening (Section 1.4.6).

Given these restrictions, we were only able to investigate the effect of the concentration of cationic surfactant on the migration of UV active anions. Figure 2.3 shows the effective mobilities (Section 2.2.3) of these anions under varying concentrations of TTAB.

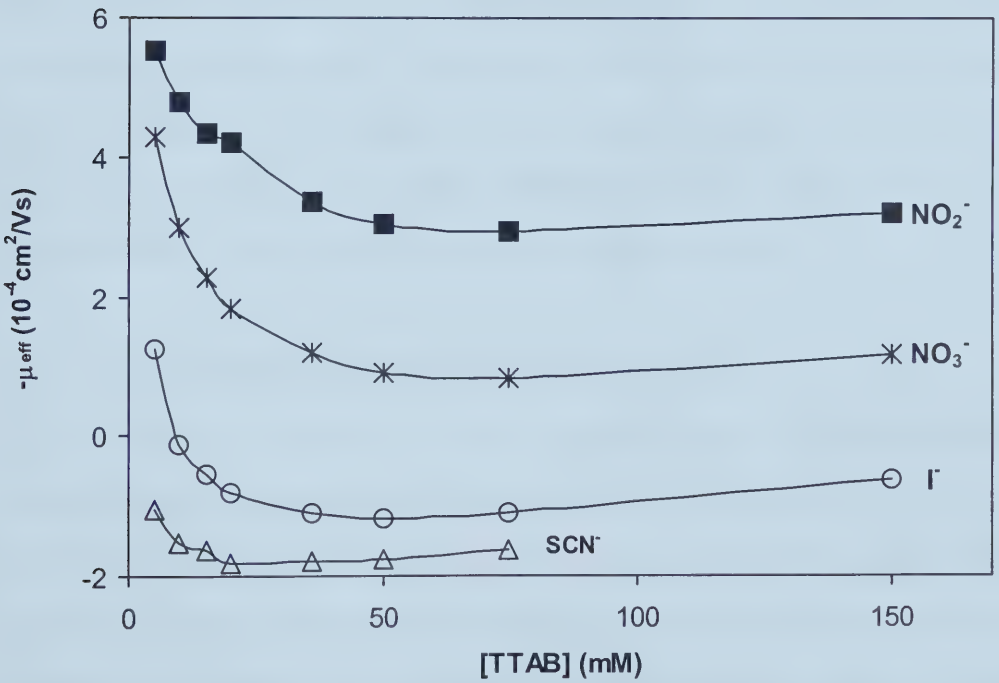


Figure 2.3 Effect of Concentration of Cationic Surfactant on the Effective Mobility of Inorganic Anions. Symbols are the experimentally determined mobilities and lines are a smoothed connection of points to show trends. Refer to Section 2.2.3 for experimental conditions.

Dramatic changes in mobility were observed up to ~15 mM TTAB. However, no significant mobility changes were observed at higher concentrations. This transition is due to the complex nature of the surfactant additive as described below.

As the concentration of TTAB increases, the number of ion association sites increases. At the same time, the concentration of bromide, a potential eluent increases proportionally whereas the concentration of the phosphate buffer, a stronger eluent stays constant at 10 mM. [44] Thus, as the TTAB concentration increases in Figure 2.3, the anions become increasingly retained by the cationic surfactant since the phosphate concentration relative to cationic surfactant decreases. At higher concentrations of TTAB, bromide becomes the more concentrated eluent, but the ratio of eluent to retentive sites remains constant. This provides a constant amount of eluent to compete with the analytes for ion association sites and thus, the effective mobilities of the analytes do not change. In summary, TTAB provides some mobility alteration, but problems restrict its use to a narrow set of experimental conditions.

2.3.2 Effect of DDAPS Concentration on Anion Mobility

The zwitterionic surfactant, DDAPS (3-(N,N-dimethyldodecylammonio)propane sulfonate, Fig. 2.1B), was added to a chromate buffer to investigate its effect on the anion mobility. In contrast to TTAB, Figure 2.2 shows that the current remains constant over the full DDAPS concentration range (3.75 to 100 mM) allowing high concentrations of surfactant to be used if desired. The use of a zwitterionic species does not contribute to the conductance of the buffer since its net charge is zero. As a result, current does not increase with concentration, inducing no additional Joule heating. Figure 2.4 shows

the effective mobilities (Sec. 2.2.4) of NO_2^-, NO_3^-, Br^-, I^-, SCN^-, F^-, Cl^-, SO_4^{2-} and $C_2O_4^{2-}$ determined in 3.75 to 120 mM DDAPS.

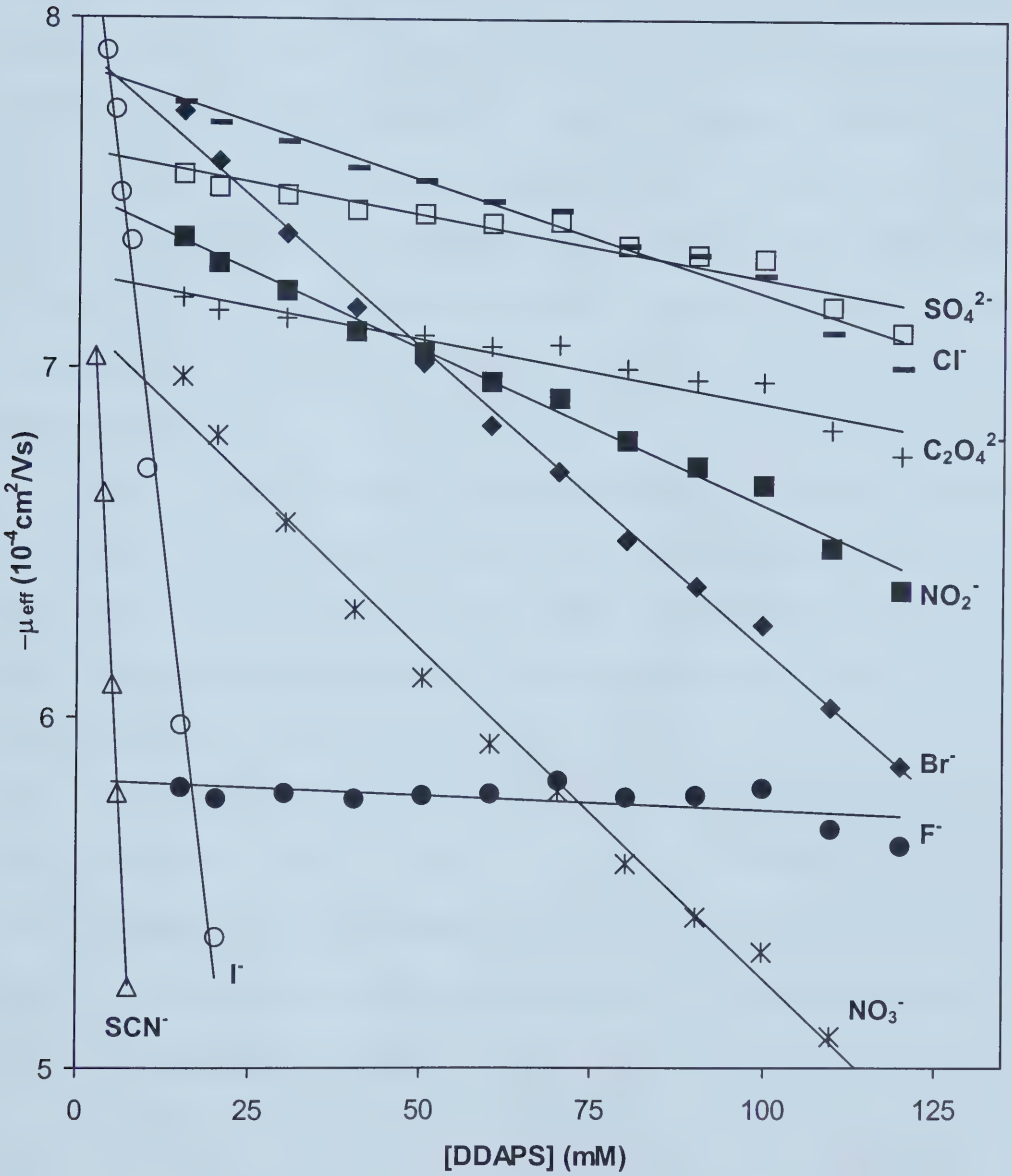


Figure 2.4 Effect of Concentration of Zwitterionic Surfactant on the Effective Mobility of Inorganic Anions. Symbols are experimentally determined mobilities and lines are best fit linear regression through data. Refer to Section 2.2.4 for experimental conditions.

When the zwitterionic surfactant is used, a dramatic change in selectivity is observed for several analytes. In particular, more polarizable anions such as thiocyanate, iodide, nitrate, and bromide exhibit substantial retarded mobility as the concentration of surfactant increases. This is consistent with the retention observed in electrostatic ion chromatography. [29, 31, 33] Furthermore, no leveling off behaviour is observed in Figure 2.4, in contrast to the TTAB behaviour shown in Figure 2.3. This demonstrates an advantage of using zwitterionic surfactants since it is possible to add the retentive phase (surfactant) without increasing the eluent strength. However, the cationic surfactant must always be accompanied by a counter-ion which will act as an eluent for the ion-exchange retention.

Figure 2.5 (on the following page) demonstrates the separation of inorganic anions using 3.75 mM and 60 mM DDAPS. At low concentrations of DDAPS, the mobility order is Br^-, Cl^-, I^-, SO_4^{2-}, NO_2^-, NO_3^-, $C_2O_4^{2-}$, SCN^- and F^-, as shown in Figure 2.5(A). This is the same as that observed for low concentrations of TTAB. [45] However, at 120 mM DDAPS, the mobility order is SO_4^{2-}, Cl^- , $C_2O_4^{2-}$, NO_2^-, Br^-, F^-, NO_3^-, I^- and SCN^- (electropherogram not shown). This differs significantly from conventional capillary zone electrophoresis. Moreover, this separation order is essentially identical to that observed in electrostatic ion chromatography ($SO_4^{2-} \approx Cl^-$, NO_2^-, Br^-, NO_3^-, ClO_3^-, I^- and SCN^-). [29] A typical electropherogram for the separation of inorganic anions using 60 mM DDAPS is shown in Figure 2.5(B).

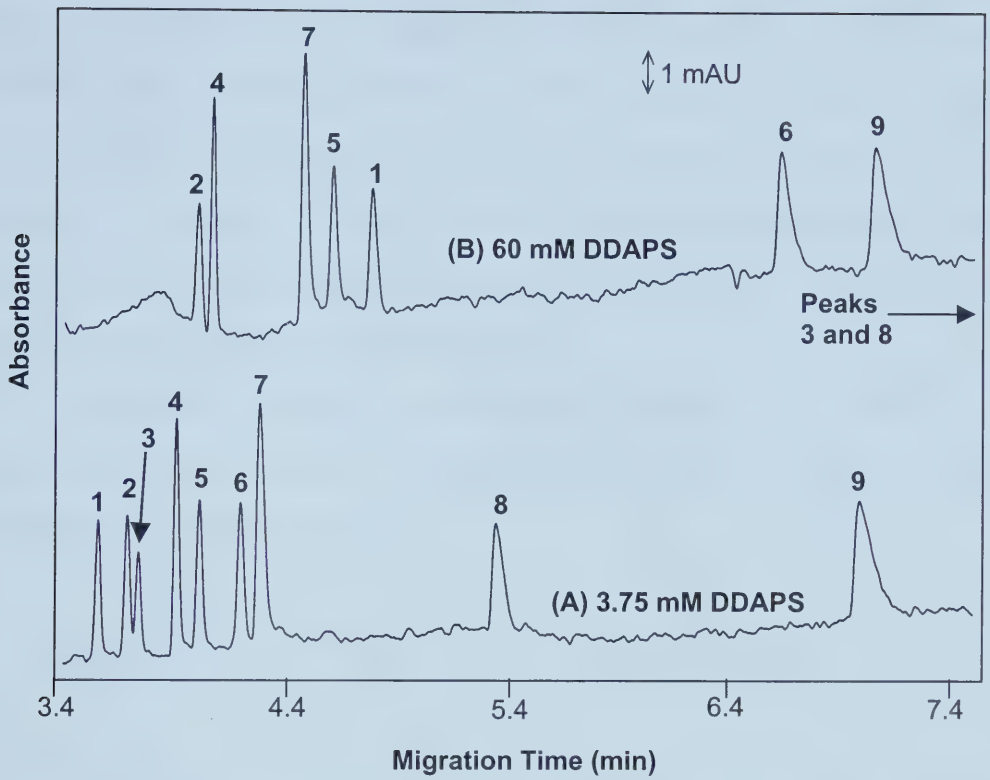


Figure 2.5 Typical Electropherograms for the Separation of Inorganic Anions using DDAPS. (A) 3.75 mM DDAPS (B) 60 mM DDAPS Peaks: (1) Bromide (2) Chloride (3) Iodide (4) Sulfate (5) Nitrite (6) Nitrate (7) Oxalate (8) Thiocyanate (9) Fluoride. Refer to Section 2.2.4 for experimental conditions.

2.3.3 Preliminary Investigation into the Nature of the Pseudo-Stationary Phase

2.3.3.1 The Importance of Micelles

In electrostatic capillary electrophoresis, the pseudo-stationary phase used to provide retention is a zwitterionic surfactant above its critical micelle concentration (cmc). As a result, it exists in solution primarily as micelles, that is, aggregates of surfactant monomers. To investigate whether micelles are required to provide retention,

a zwitterionic surfactant below its cmc was probed for its ability to retain anions. Since a shorter hydrophobic chain will increase the cmc of the surfactant, the octyl-form of DDAPS was used, namely 3-(N,N-dimethyloctylammonio)propane sulfonate (Zwittergent 3-08, Fig. 2.1C). At concentrations below the cmc (330 mM), [38] it will exist solely in the monomer form instead of micelles. The use of a zwitterionic surfactant with the same head group as DDAPS will allow explicit study of the importance of micelle formation without interference from head group chemistry.

The effective mobility of the anions was measured as a function of the concentration of Zwittergent 3-08. Experimental details are given in Section 2.2.5 and the results are shown in Figure 2.6.

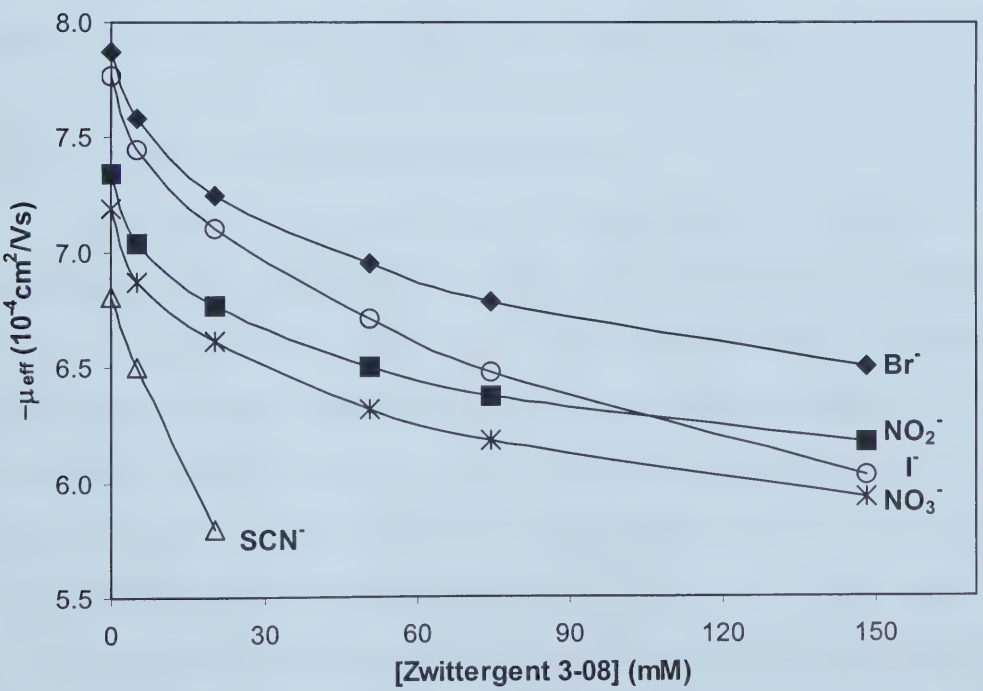


Figure 2.6 Effect of Concentration of Zwitterionic Surfactant (below its cmc) on the Effective Mobility of Inorganic Anions. Symbols are experimentally determined mobilities and lines are smoothed connection of points to show trends. Refer to Section 2.2.5 for experimental conditions.

No dramatic selectivity changes are observed with the addition of 5 to 150 mM zwitterionic surfactant monomers to the buffer. A decrease in mobility for all anions is observed. This can be attributed to ion pair formation with the positive functionality on the surfactant head group. [46] The formation of ion pairs also explains the significant decrease in thiocyanate mobility and the selectivity change between iodide and nitrite. Kaneta et al. [17] observed a similar cross-over while using cetyltrimethylammonium chloride (C_{16}TAC) below its cmc. In fact, the ion association constant of iodide was calculated to be at least five times greater than other anions such as nitrate or bromide.

Only small changes in mobility are observed with the use of a zwitterionic surfactant below its cmc. These effects are insignificant compared to the dramatic selectivity changes obtained by using DDAPS above its cmc (Figure 2.4). Therefore, the presence of micelles is required for the prolonged retention of anions.

2.3.3.2 The Importance of Head Group Chemistry

The importance of micelles in the alteration of selectivity of inorganic anion separations has been demonstrated. Now, the role of the zwitterionic head group will be studied. Cationic surfactants such as TTAB have shown little ability to alter selectivity (Figure 2.3). Furthermore, anionic surfactants such as sodium dodecyl sulfate (SDS) do not affect the mobility of inorganic anions since anions are repelled by the negative head group. However, the utility of nonionic surfactants in altering the selectivity of inorganic anion separations has not yet been studied.

Brij 35 (Fig. 2.1D), a polyoxyethylene (23) dodecyl ether, is a nonionic surfactant with a cmc of 0.92 mM. [38] The effect of Brij 35 (at concentrations above its cmc) on the

effective mobility of anions was investigated. The experimental conditions are given in Section 2.2.6 and the results are presented in Figure 2.7.

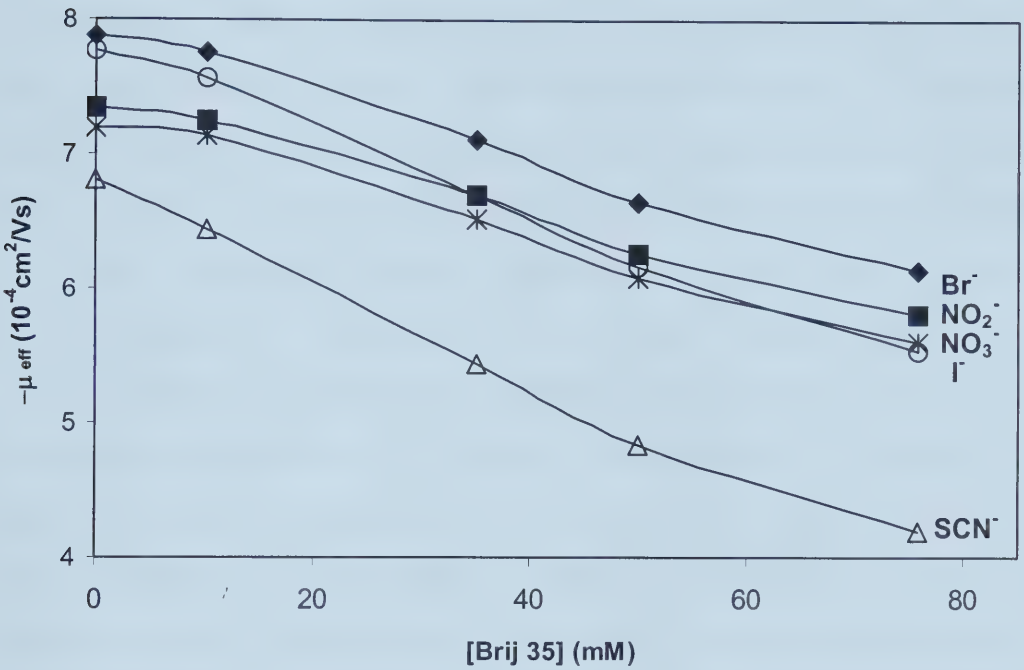


Figure 2.7 Effect of Concentration of Nonionic Surfactant on the Effective Mobility of Inorganic Anions. Symbols are experimentally determined mobilities and lines are smoothed connection of points to show trends. Refer to Section 2.2.6 for experimental conditions.

The highest concentration of Brij 35 studied was 75 mM due to difficulty in dissolving higher concentrations. The general effects on mobility are similar to those observed with the use of Zwittergent 3-08. However, the decrease in effective mobility observed with Brij 35 (Fig. 2.7) is generally more pronounced than observed in Figure 2.6 for Zwittergent 3-08. This is due in part to the increased viscosity of the Brij 35 solutions. A viscosity increase will decrease mobility according to the Hückel equation (eqn 1.5).

However the effects in Figure 2.7 are not solely due to viscosity. The mobility of iodide decreases such that it migrates later than nitrate and nitrite in 75 mM Brij 35. Kaneta et al. [17] have demonstrated that inorganic anions can partition into the core of positively charged micelles. Iodide has a higher distribution coefficient than the other inorganic anions studied (bromide, bromate, nitrate and iodate), thus explaining the cross-over observed here. For the sake of this study, the important observation is that increasing the concentration of nonionic surfactant failed to produce dramatic selectivity changes such as those observed with DDAPS. Therefore, the zwitterionic nature of the head group is an integral part of the ability of DDAPS to alter anion mobility.

In conclusion, an effective pseudo-stationary phase must have both a zwitterionic head group and a hydrophobic tail. To provide substantial alteration of selectivity, the zwitterion must be used at concentrations above its cmc. This ensures the formation of micelles in solution. However, zwitterionic surfactants with very low cmc values are undesirable since their solubility and thus, applicability, is limited to low concentrations. Finally, the head group must remain zwitterionic over a large pH range. This means that the basic and acidic groups must have very high and low pK_a values, respectively.

2.3.4 Mechanism of Retention

The importance of both micelles and a zwitterionic head group in electrostatic CE has been demonstrated in Section 2.3.3. At this point, a mechanism is required to explain the observed anion retention. The experimental conditions used in this study are similar to those used in electrostatic ion chromatography. The DDAPS zwitterionic surfactant used here is merely a shorter chain than the C14 surfactant used to coat ODS columns in electrostatic IC. [25-27, 29] Furthermore, the migration order observed in this

study is exactly the same as the elution order observed in electrostatic IC. [29] These factors have led us to believe that the mechanism for electrostatic ion chromatography also explains the behaviour observed in Figure 2.4. The primary difference between the two methods is where in the column retention of analytes occurs. In electrostatic IC, the analyte interacts with the zwitterionic micelle which is adsorbed to an ODS phase at the wall. Conversely, in electrostatic CE, the analyte is retained by the zwitterionic micellar phase in bulk solution.

Several mechanisms have been offered to explain the retention observed in electrostatic IC. In 1994, Hu et al. [26] proposed a simultaneous electrostatic attraction and repulsion between analyte ions and the oppositely charged groups on the zwitterion. In this way, retention was governed by the ability of the analyte to form ion pairs with the zwitterion. Four years later, Hu and Haddad [27] postulated that an electrostatic double layer formed as a result of accumulation of cations and anions from the mobile phase electrolyte around the doubly-charged zwitterionic head group. Anion retention was based on the propensity of the analyte to form ion pairs with this double layer. However, these mechanisms failed to explain all of the behaviour observed in electrostatic IC.

The recent paper by Haddad and coworkers [34] presents an improved, comprehensive mechanism for the retention observed in electrostatic IC. It is believed that this mechanism also explains the selectivity changes observed this work. The revised mechanism encompasses two different effects, ion exclusion and chaotropic interaction, which are illustrated in Figure 2.8 on the following page.

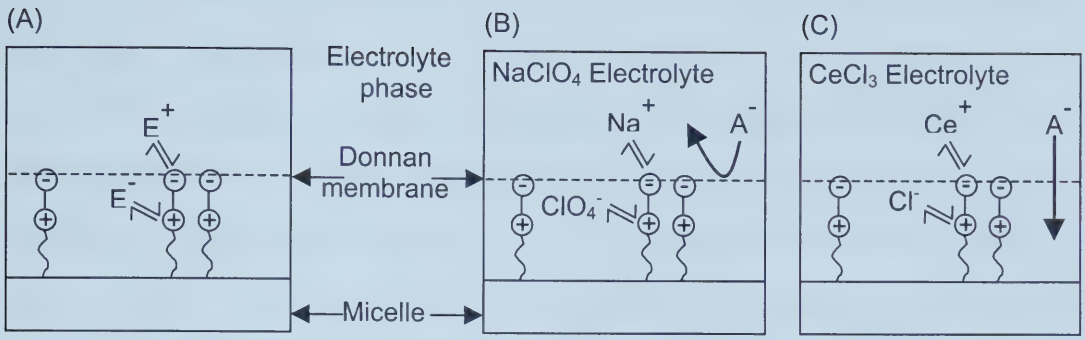


Figure 2.8 Schematic Summary of the Proposed Electrostatic IC mechanism. (A) Establishment of the Donnan membrane (B) Use of a $NaClO_4$ electrolyte (C) Use of $CeCl_3$ electrolyte. Adapted from *Anal. Chem.* **2001**, 73, 3022-3027.

Ion exclusion arises from the establishment of a Donnan membrane around the charges of the zwitterionic head group. The sulfonate group on the outer part of the head group possesses a negative charge which repels analyte anions. However, the degree of repulsion depends upon the strength of the interaction between electrolyte cations (E^+) and the head group. Strong interaction by electrolyte cations will decrease the effective negative charge and cause less repulsion of the analyte anions. However, strong association by the electrolyte anion (ClO_4^- in Figure 2.8B) will increase the negative charge of the zwitterionic phase, leading to more electrostatic repulsion of the analyte anion (A^-), as shown in Figure 2.8 (B).

Once analyte anions penetrate the repulsive barrier, they interact with the positive quaternary ammonium functional group. At this point, separation selectivity follows the order of increasing polarizability or chaotropic character of the analyte anion. Chaotropic ions are those which disrupt the water structure surrounding them, thereby causing an increase in entropy. [47] The Hofmeister series is an ordering of ions based on their chaotropic character as follows: $IO_3^- < F^- < SO_4^{2-} < Cl^- < Br^- < I^- < ClO_4^- < SCN^-$

where SCN^- is the most chaotropic. [34, 47, 48] This anion order mimics the retention trends observed in electrostatic IC, thus offering support for the proposed mechanism.

Previous work in our group investigated the effect of polarizable anions on the EOF generated in a capillary coated with a sulfobetaine zwitterionic surfactant. [49] The buffer anion was varied (ClO_4^-, I^-, Br^-, Cl^-, SO_4^{2-}) while keeping the cation (K^+) constant to test the effect of the anion on EOF. An increase in buffer concentration caused an increase in EOF when zwitterionic surfactant was used. The magnitude of the EOF shift increased in the order: $SO_4^{2-} < Cl^- < Br^- < I^- < ClO_4^-$. This follows the same order as the Hofmeister series given above. According to the von Smoluchowski equation for EOF (eqn 1.7), a larger EOF indicates a more negative ζ potential. This increase in negative potential can be explained by the establishment of a Donnan membrane. [34] Perchlorate is one of the more chaotropic ions and interacts strongly with the quaternary ammonium group on the zwitterionic surfactant. However, the cation used in Baryla and Lucy's EOF studies (K^+) is not chaotropic. [47] Therefore, it would not interact with the sulfonate group. A negative layer would then be established which induces a more negative potential and increased EOF. This argument demonstrates that the electrostatic IC mechanism can be successfully applied to capillary electrophoretic separations.

The electrostatic IC mechanism also explains the retention of inorganic anions observed in Figure 2.4. Since the same type of zwitterionic surfactant (DDAPS, Fig. 2.1B) is used as in the studies of Cook et al., [34] the formation of a Donnan membrane is likely. Furthermore, the buffer anion, phosphate, is rated low in the Hofmeister chaotropic series. [47] Its weak association with the ammonium group will impart very little negative repulsion on incoming analytes. This is also evidenced by the fairly constant EOF produced with increasing concentration of phosphate in a zwitterionic surfactant

coated capillary. [49] In contrast to the increasing EOF exhibited with perchlorate, phosphate results in a constant low zeta potential. [49] Thus, analyte anions can easily penetrate the electrostatic barrier and be retained according to their chaotropic nature. The migration order of anions at high concentrations of DDAPS is the same as the elution order in electrostatic IC, [29] further supporting a common mechanism.

It is further thought that anions interact with the micelles primarily in bulk solution, as opposed to the capillary wall, since peak shapes are generally symmetric. Interactions with the micelles at the walls would cause tailed peaks due to resistance to mass transfer of analyte from the bulk solution to and from the capillary wall. These tailed peaks are evident in open tubular capillary electrochromatography and still remain to be one of its disadvantages.[50]

2.3.5 Determination of Anions in Seawater

Capillary zone electrophoresis works best with low conductivity samples. As a result, seawater has posed a challenging matrix for CE. First, the high conductivity of seawater causes electrodispersion within the sample zone (Section 1.4.4), resulting in severe distortion and broadening of the analyte peaks. Second, the mobility of chloride is similar to that of anions of interest, making separation difficult. Previous CE separations of bromide, iodide, nitrate, nitrite and thiocyanate were performed using chloride as the electrolyte to reduce matrix effects in direct UV detection. [51] Recently, Fukushi et al. [52] used artificial seawater as the electrolyte solution in CE to determine bromide, nitrite and nitrate in seawater. However, the high current generated, 330 μA, is a drawback. Electrostatic ion chromatography has also been demonstrated to be effective for the determination of bromide, nitrate and iodide in seawater by using dilute

seawater as the eluent. [53] Therefore, we will explore the use of electrostatic capillary electrophoresis for the determination of anions in seawater.

The seawater matrix was diluted 1:5 to minimize electrodispersion in the sample zone caused by the high concentrations of chloride. Based on Figure 2.4, 10 mM DDAPS should separate nitrate, nitrite and iodide from the seawater matrix, while still eluting iodide in a reasonable time. Figure 2.9(A) shows the separation of NO_2^-, NO_3^- and I^- in 1:5 diluted seawater.

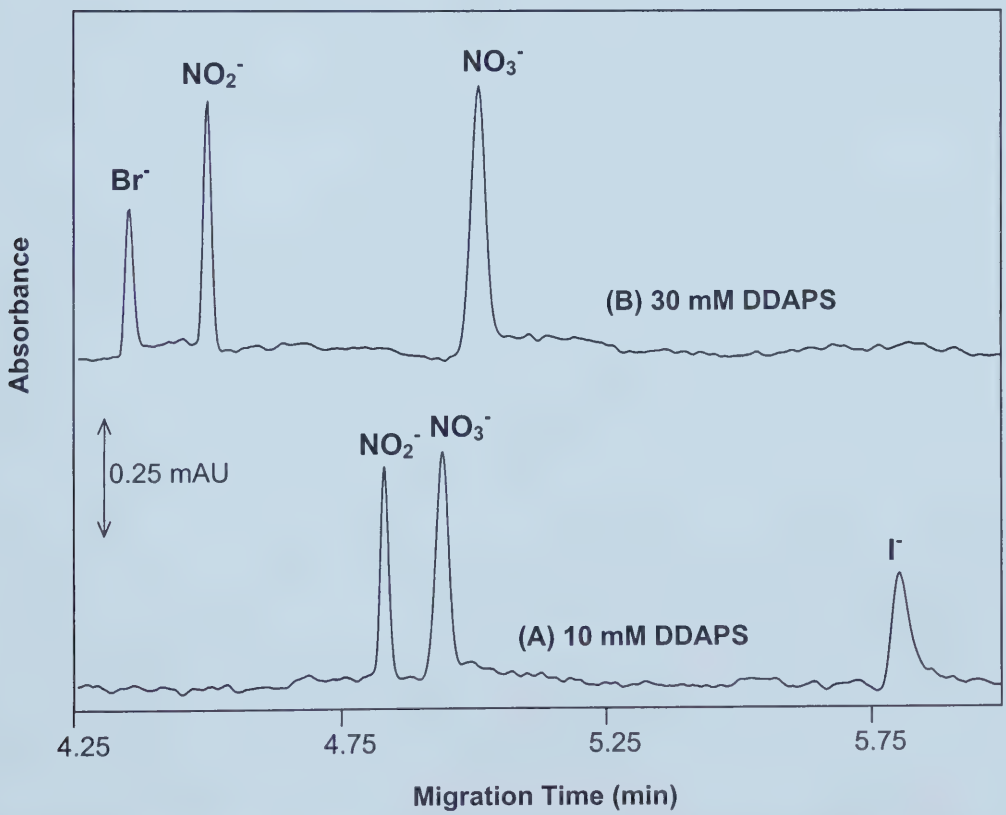


Figure 2.9 Determination of Inorganic Anions in Artificial Seawater (A) 10 mM DDAPS in 10 mM phosphate pH 8.0; 150 μM sample (B) 30 mM DDAPS in 10 mM phosphate pH 8.0; 75 μM sample. Refer to Section 2.2.7 for other experimental conditions.

Bromide could not be determined using the conditions in Figure 2.9(A) as it still co-eluted with the large chloride matrix peak and could not be detected. If instead, 30 mM DDAPS was added to the phosphate buffer, the bromide can be removed from the chloride matrix, as shown in Figure 2.9(B). The ability to move peaks selectively demonstrates the flexibility of this technique. However, this higher concentration of DDAPS results in excessively long migration times for iodide.

Calibration graphs for all anions were linear from 0.05 to 1.0 mM with intercepts equal to zero at the 95% confidence interval. A sample calibration graph for nitrite is given in Figure 2.10.

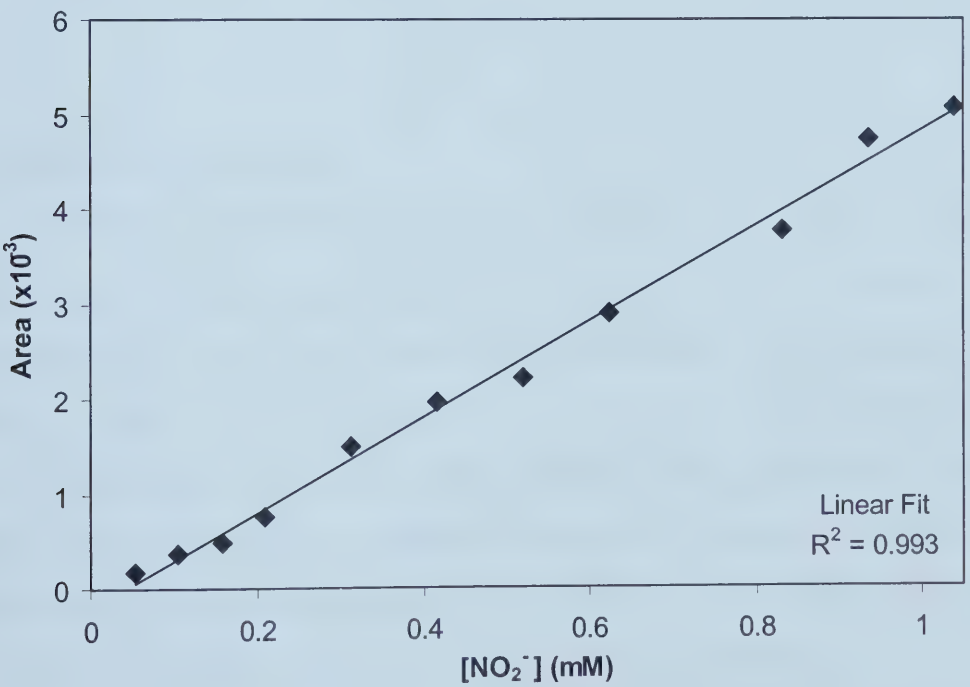


Figure 2.10 Calibration Graph for Quantification of Nitrite in Artificial Seawater using 10 mM DDAPS in 10 mM Phosphate Buffer pH 7.21. Symbols are experimental data and line is best fit linear regression through data. Refer to Section 2.2.7 for experimental conditions.

Table 2.1 lists the detection limits, correlation coefficients (r^2) and RSD values for the migration times and peak areas. Timerbaev et al. achieved detection limits of 1-2 μM in 1:10 dilute seawater at 200 nm using a chloride background electrolyte. [51]

Table 2.1 Analysis Data for the Determination of Inorganic Anions in Seawater (Figure 2.9).

Anion	Correlation Coefficient (r^2)	Peak Area RSD (%)	Migration Time RSD (%)	Detection Limit in 1:5 Diluted Seawater (μM)
Iodide[a]	0.955	10.3	0.64	11
Nitrate[b]	0.991	7.2	0.74	11
Nitrite[b]	0.993	3.1	0.95	5
Bromide[b]	0.990	3.5	0.82	7

[a] Experimental conditions are the same as in Figure 2.9(A) except a 100 μM sample was used. [b] Experimental conditions are the same as in Figure 2.9(B).

2.4 CONCLUDING REMARKS

The addition of zwitterionic surfactants to the background electrolyte allows one to change the selectivity of inorganic anion separations from that of capillary zone electrophoresis to the orthogonal selectivity of electrostatic ion chromatography. The choice of zwitterionic surfactant concentration allows the analyst to decide the migration order of the analytes, minimizing the impact of interfering peaks. This technique has the added bonus of not inducing any additional Joule heating. Electrostatic CE has been successfully applied to the separation of inorganic anions in seawater. The flexibility and ease of this technique provides much opportunity for future applications.

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CHAPTER 3: USE OF Z1-METHYL IN CE SEPARATIONS OF PROTEINS

3.1 INTRODUCTION

The recent boom in genomics and analytical biotechnology generated a pressing demand for fast, efficient and automated methods to separate biomolecules such as proteins. Capillary electrophoresis has filled this niche commendably, in spite of the many difficulties. In theory, protein separations should yield efficiencies in excess of a million plates. This is due to the low diffusion coefficient of macromolecules and the ability to use high field strengths as represented in equation 3.1. [1]

$$N = \frac{(\mu_{app} + \mu_{EOF})V_{appl}}{2D} \qquad (3.1)$$

where N is the number of theoretical plates, μ_{app} is the electrophoretic mobility of the analyte, μ_{EOF} is the mobility of the electroosmotic flow, V_{appl} is the applied voltage and D is the molecular diffusion coefficient of the solute in the zone. In practice, the efficiencies achieved are substantially reduced and separations are plagued by peak tailing, irreproducibility and poor recoveries. [2] These phenomena are a result of protein adsorption onto the bare fused silica capillary wall. At pH values nearing the pK_a of the silanols, they become deprotonated resulting in a negative charge at the wall. At a pH below the isoelectric point of the protein, it has a net positive charge and is electrostatically attracted to the capillary wall. Adsorbed proteins change the zeta potential at the wall causing the EOF to vary along the length of the capillary. In addition, reversibly adsorbed proteins may desorb and elute at a different position than the unretained protein, producing artifacts in the electropherogram. [1]

Various techniques have been used to reduce the undesirable effects of wall adsorption. In a bare silica capillary, endeavours to minimize Coulombic attraction between the protein and the wall have achieved success. Low pH has been used to protonate the silanol groups [2] and conversely, high pH has been used to ensure all proteins are negatively charged. [3] Unfortunately, extreme pH may denature proteins, reduce the charge diversity and thus, selectivity [1] and eliminate a key way to adjust EOF.

Surfactants have been utilized to coat the silica wall and reduce the number of adsorption sites available. Nonionic surfactants such as Brij 35 have shown success by adsorbing their hydrophobic tail to derivatized silanols, leaving the hydrophilic head group to repel the protein. [4] Cationic surfactants such as cetyltrimethylammonium bromide (C_{16}TAB) and dodecyldimethylammonium bromide (DDAB) produce a wall charge reversal thereby allowing the efficient separations of basic proteins, but not acidic proteins. [5-7] Finally, the simultaneous separation of cationic and anionic proteins has been accomplished by coating the wall with a zwitterionic surfactant possessing a sulfobetaine headgroup. [8, 9]

An additional way to diminish protein interactions with the wall is to shield the silanol groups with an applied polymer layer. Covalently attached or cross-linked polymers were first introduced by Hjertén [10] and since then, have shown much promise for the analysis of basic and acidic proteins. [11-14] These coatings have the advantage of removing the wall modifier from the running buffer. However, application procedures may be time consuming and laborious. Noncovalent polymer coatings formed by electrostatically adsorbed polycations are more simple to apply, but are limited to separating cationic proteins. [14-16]

Another approach to minimize silanol effects is to add competing ions such as amines, salts or zwitterions to the background buffer. Amines such as diaminoalkanes function by ion pairing with the negative silanol groups, thereby competing with the protein for these positions. [17] High concentrations of salts such as potassium increase the ionic strength of the buffer and also compete for cation-exchange sites on the surface. However, they have the disadvantage of increasing conductivity and Joule heating. [18-20] To remedy this problem, zwitterionic additives can be used which do not increase the conductivity of the buffer. This allows the use of higher voltages and concentrations of additive.

Bushey and Jorgenson [19] investigated five zwitterions for their efficacy in preventing protein adsorption and concluded that trimethylglycine hydroxide (betaine) was the most successful. Sun and coworkers [21] demonstrated that trimethyl-ammoniumalkyl sulfonate zwitterions increases protein efficiency and mobility. The propyl-form of this zwitterion has been marketed by Waters as the Accupure Z1-Methyl additive for prevention of protein-wall interactions. [22] Fang and Sun [21] demonstrated a 20-fold increase in protein efficiency with the addition of 2.0 M Z1-Methyl to the buffer. Guzman et al. [23] obtained an improvement in peak area reproducibility in the separation of monoclonal antibodies with the use of 0.025 M Z1-Methyl. Z1-Methyl has also been used to prevent adsorption in the determination of ricin, a toxic glycoprotein; [24] free fatty acids; [25] deamidation products in insulin solutions; [26] and monoclonal proteins. [27] Although Z1-Methyl has been added to the buffer to provide more efficient protein separations, its mechanism has not been made clear. This research studies the effect of Z1-Methyl on protein adsorption and electroosmotic flow and provides an explanation for its intriguing behaviour.

3.2 EXPERIMENTAL

3.2.1 Apparatus

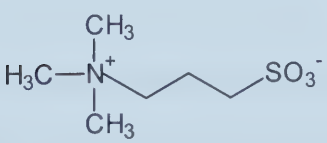
Experiments were performed using a Beckman P/ACE 2100 system (Beckman Instruments, Fullerton, CA) with UV absorbance detection using a rise time of 1 s. Data acquisition (5 Hz) and control was performed using P/ACE Station software for Windows 95 (Beckman) on a Pentium 120 MHz microcomputer. Untreated silica capillaries (Polymicro Technologies, Phoenix, AZ) with an inner diameter of 50 μm, outer diameter of 365 μm, and total lengths of 47 cm or 27 cm (40 cm or 20 cm to the detector, respectively) were used. Capillary lengths were measured after use to determine accurate values. Fresh capillaries were used for each new buffer system to avoid hysteresis effects. New capillaries were pre-treated with a high pressure (138 kPa) rinse using 0.1 M sodium hydroxide (BDH, Toronto, ON) for 5 to 10 minutes. Prior to each run, the capillary was rinsed at high pressure with 0.1 M sodium hydroxide for 1 to 2 min, water for 1 to 2 min and buffer for 2 to 3.5 min. Higher rinse times were used for the longer capillary and lower values were used for the shorter capillary. Six electrophoretic runs were performed in each new buffer prior to the acquisition of real data. This technique equilibrates the capillary under voltage in the new buffer and provides more stable and reproducible EOF. [28] In all experiments, the capillary was thermostated at 25 ^{0}C using liquid coolant.

3.2.2 Reagents

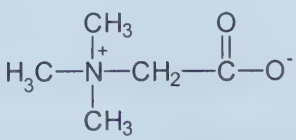
All solutions were prepared in Nanopure (18 MΩ) water (Barnstead, Chicago, IL). Buffers were prepared from stock solutions of reagent grade orthophosphoric acid (BDH, Toronto, ON) or glacial acetic acid (Anachemia, Rouses Point, NY) with sodium

hydroxide (BDH) to adjust the pH unless otherwise stated. The pH values were measured using a Corning digital pH meter model 445 (Corning, Acton, MA) calibrated immediately prior to use. A solution of mesityl oxide (Aldrich) for detection at 254 nm or benzyl alcohol (Aldrich) in water for detection at 214 nm was used as the neutral EOF marker. Methylamine (40% by weight, Matheson, Coleman and Bell, Norwood, OH) and acetic acid (Anachemia) were diluted to make sample solutions for titration. Hydrochloric acid titrant was made from reagent grade hydrochloric acid (Anachemia) and sodium hydroxide titrant was made from sodium hydroxide pellets (BDH). Didodecyldimethylammonium bromide, (DDAB) (Aldrich, Milwaukee, WI) and trimethylammoniumpropane sulfonate (Z1-Methyl) (Waters, Milford, MA) were used as received. Waters is gratefully acknowledged for the kind gift of Z1-Methyl. The structures of these two compounds as well as the betaine zwitterion used by Jorgenson [19] are shown in Figure 3.1.

(A) Z1-Methyl, pK_a of SO_3^- group ≈ 1.5 [29]



(B) Betaine, pK_a of COO^- group = 1.83 [29]



(C) DDAB, critical vesicle concentration = 35 μM [30]

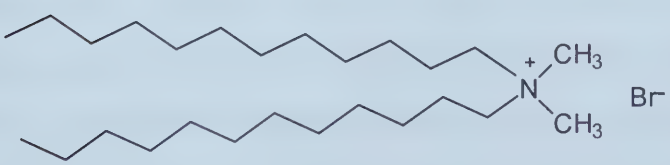


Figure 3.1 Structures of Zwitterions and Surfactants

Samples of 0.15 to 0.5 mg/mL (3.5 to 35 μM) protein in water were prepared from standards (Sigma, St. Louis, MO) of lysozyme (chicken egg white), myoglobin (horse skeletal muscle), α-lactalbumin (bovine milk), albumin (chicken egg, ovalbumin), and bovine serum albumin (BSA) without further purification. Table 3.1 summarizes the physical properties of the proteins used.

Table 3.1 Physical Properties of Proteins [31]

Protein	SwissProt #	Molecular Weight (g/mol)	pI	Charge at pH 7.2
Lysozyme	P00698	14300	11	Positive
Myoglobin	P02188	17000	7	Negative
α-lactalbumin	P00711	14200	4.8	Negative
BSA	P02769	66400	5.6	Negative
Ovalbumin	P01012	43000	5.2	Negative

Samples of 0.5 mM inorganic anions in water were prepared from sodium nitrite (BDH), potassium nitrate (BDH), potassium bromide (Fisher Scientific, Fair Lawn, NJ), sodium iodide (BDH) and potassium thiocyanate (BDH) without further purification.

3.2.3 Relative Viscosity Measurements

Mobilities were corrected for viscosity effects in the more viscous Z1-Methyl solutions. The viscosities of the buffers containing Z1-Methyl were measured relative to the buffer with no additive. Benzyl alcohol was hydrodynamically injected (2 s at 3.45 kPa) into a capillary filled with test buffer and the peak was transported past the detector (214 nm) by applying low pressure. The relative viscosity correction was denoted by the ratio of the benzyl alcohol elution times in buffer containing Z1-Methyl and pure buffer. Relative viscosity measurements were also obtained for the various concentrations of

phosphate buffer and corrected with respect to the 100 mM phosphate buffer. The viscosity correction factors are summarized in Table 3.2.

Table 3.2 Viscosity Correction Factors

[Phosphate] (mmol/L)	[Z1-methyl] (mmol/L)	Correction Factor Relative to 10 mM phosphate	Correction Factor Relative to 100 mM phosphate
100	0		1.000
100	200		1.054
100	400		1.127
100	600		1.204
100	800		1.299
100	1000		1.398
75	0		0.984
100	0		1.000
120	0		1.007
150	0		1.026
175	0		1.041
10	0	1.000	
10	25	1.008	
10	49	1.016	
10	101	1.033	
10	200	1.066	
10	299	1.098	
10	398	1.131	
10	500	1.164	
10	1008	1.405	
10 (acetate)	1000	1.402	

3.2.4 Effective Mobility of Proteins

The effect of ionic strength on protein mobility was investigated by varying the phosphate buffer concentration from 50 to 175 mM in 1.0 M Z1-Methyl at pH 7.21. Similarly, the effect of Z1-Methyl (Fig. 3.1A) on protein mobility was studied by varying its concentration from 200 to 1000 mM in 100 mM phosphate buffer (pH 7.21). The mobility was also studied in 10 mM phosphate (pH 7.21) with 1.0 M Z1-Methyl added. In

all cases, a mixture of 0.5 mg/mL lysozyme and 0.5 mM benzyl alcohol was injected using low pressure (3.45 kPa) for 2 s into a 27 cm capillary. The protein was separated from the neutral marker with an applied potential of 5.0 to 7.5 kV (normal EOF from anode to cathode). Direct UV detection was used at 214 nm. Mobilities become dependent upon voltage only at field strengths greater than 10^4 V/cm (Wien effect). [32] Hence, the mobilities are independent of applied voltage for the field strengths used here.

Ohm's law plots were performed for each buffer system to confirm that Joule heating was not a concern at the voltages used. A range of voltages bracketing the desired value were applied in random order for 1.5 min and the current was recorded after 1.0 min. Correlation coefficients were computed from a linear least squares fit (MS Excel 97) of the plot of current versus voltage. Values of 0.999 or greater were considered acceptable for linearity.

The apparent mobility, μ_{app}, of the cationic protein was determined from the migration time under constant voltage conditions using the equation

$$\mu = \frac{L_T L_d}{t V_{appl}} \qquad (3.2)$$

where L_T is the total capillary length, L_d is the effective capillary length to the detector, t is the migration time and V_{appl} is the applied voltage. This equation was also applied to calculate the mobility of the EOF, μ_{EOF}, by inserting the migration time of the neutral marker. Ultimately, the effective mobility of the protein, μ_{eff}, corrected for EOF, was calculated using the equation

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \qquad (3.3)$$

The average of three mobilities from replicate runs is corrected for viscosity effects and reported.

3.2.5 Protein Separations

The separation of a protein mixture was accomplished in 100 mM phosphate buffer at pH 7.21 with 1.0 M Z1-Methyl added. A mixture of 0.25 mg/mL lysozyme, 0.15 mg/mL ovalbumin, 0.15 mg/mL myoglobin and 0.15 mg/mL α-lactalbumin was injected into a 27 cm capillary using low pressure for 4 s. The applied voltage was 7.5 kV (normal EOF) and direct detection was used at 214 nm. Efficiencies were calculated using the Foley–Dorsey equation for asymmetric peaks, [33, 34]

$$N \approx \frac{41.7(t/w_{0.1})^2}{(B/A+1.25)} \qquad (3.4)$$

where N is the number of theoretical plates, t is the analyte migration time, $w_{0.1}$ is the peak width (A+B) at one-tenth-height, and B/A is the asymmetry factor as illustrated in Figure 3.2.

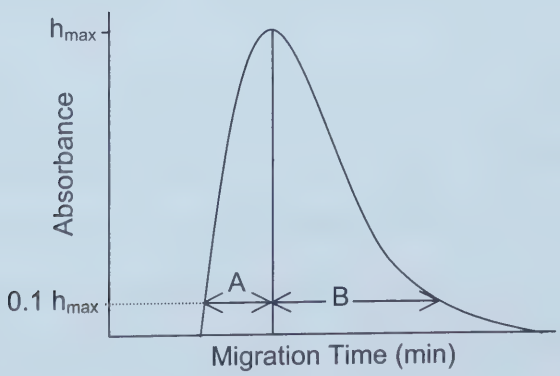


Figure 3.2 Foley-Dorsey Asymmetry

3.2.6 Effective Mobility of Anions

The influence of Z1-Methyl on the effective mobilities of five inorganic anions was studied in 100 mM phosphate (pH 7.21) with 0 mM and 1000 mM Z1-Methyl added. The three-peak method of Williams and Vigh [35] was used since the mobilities of the anions were closely matched and opposite in direction to the high EOF, causing excessively long run times. In the Williams-Vigh procedure, a mixture of 0.5 mM anion and 1.0 mM benzyl alcohol was injected into a buffer-filled 47 cm capillary for 2 s at 3.45 kPa. The band was pushed into the capillary using low pressure for 6 min. A constant voltage of 5.0 kV was applied from anode to cathode for 6 min. During this time, the neutral marker moves toward the cathode with a mobility, μ_{EOF}. The anion moves towards the anode with its electrophoretic mobility, μ_{eff}, but is simultaneously dragged towards the cathode by the EOF. Next, a solution of benzyl alcohol was injected for 2 s and the peaks were swept past the detector (214 nm) using low pressure. The effective mobility of the anion, μ^{A}_{eff}, was finally calculated using the equation [35]

$$\mu^{A}_{eff} = \frac{L_T L_d (t_A - t_{N_1})}{V_{appl}(t_{N_2} + t_{inj}/2)(t_{migr} - t_{ramp-up}/2 - t_{ramp-down}/2)} \qquad (3.5)$$

where t_A, t_{N1} and t_{N2} are the times required to push the anion, first neutral marker and second neutral marker, respectively, past the detector. V_{appl} is the applied voltage; t_{inj} is the injection time; t_{migr} is the time the voltage is applied and $t_{ramp\text{-}up}$ and $t_{ramp\text{-}down}$ are the times required to linearly change the applied potential between zero and V_{appl} (10.2 s for this study). The average mobility of three replicates is corrected for viscosity effects and reported.

The effective mobility of a negatively charged protein, bovine serum albumin (BSA), was studied in 100 mM phosphate (pH 7.21) with 0 mM and 1000 mM Z1-Methyl added. The conventional method was used whereby a mixture of 0.5 mg/mL BSA and 1 mM benzyl alcohol was injected for 3 s into a 47 cm capillary. The two peaks were separated under a constant voltage of 10 kV and detected (214 nm) near the cathode. Equations 3.2 and 3.3 were used to calculate the effective mobility of the protein. The average mobility of five replicates is corrected for viscosity effects and reported.

3.2.7 Effect of Z1-Methyl on EOF

The concentration of Z1-Methyl was varied from 10 to 1000 mM in a 10 mM phosphate buffer (pH 7.21) to study the zwitterion's effect on EOF. Mesityl oxide was injected into a 47 cm capillary for 2 s using low pressure. A voltage of 20 kV was applied for a sufficient time to move the neutral marker past the detector (254 nm). Values of EOF were calculated from eqn 3.2. The average EOF of two replicates is corrected for viscosity effects and reported.

3.2.8 Determination of pK_a Values

The pK_a values of methylamine and acetic acid were determined. In the first case, 10 mL of 20 mM methylamine in water was titrated with a 50 mM hydrochloric acid solution from a Class A 10 mL buret. The volume of acid added was recorded along with the corresponding pH obtained from a Corning digital pH meter every 0.1 to 0.4 mL depending on the region of the titration curve. The titration curve was plotted as pH versus the volume of titrant added. The buffered region was fitted to the Henderson-Hasselbalch equation. [36] MS Excel Solver was used to determine the value of the pK_a

which minimized the y-residual or pH residual. The pK_a of a 20 mM methylamine solution containing 0.50 M Z1-Methyl was also measured. The pK_a of an acid was determined in a similar experiment by titrating a 20 mM solution of acetic acid in water with 50 mM sodium hydroxide. The experiment was repeated for the acetic acid solution containing 0.50 M Z1-Methyl.

3.2.9 Effect of Buffer Cation on EOF with Z1-Methyl

The effect of the buffer cation on the EOF increase caused by Z1-Methyl was investigated. EOF was measured in 10 mM phosphate buffer (pH 7.21) prepared from phosphoric acid with sodium hydroxide (BDH), potassium hydroxide (BDH) and lithium hydroxide (Aldrich) to adjust the pH. The capillary was also pre-conditioned with the corresponding 0.1 M hydroxide salt. Mesityl oxide was injected (1 s at 3.45 kPa) into a 27 cm capillary and 10 kV was applied to elute the EOF marker. The experiment was repeated with 0.25 M Z1-Methyl added to the buffer. The average EOF of two replicates is corrected for viscosity effects and reported.

3.2.10 True Effective Mobility of Lysozyme

To obtain a true effective mobility, lysozyme was separated from the EOF marker in a capillary which was coated to eliminate protein adsorption effects. A 27 cm capillary was pre-treated with 0.1 mM DDAB (Fig. 3.1C) for 5 min and then re-coated for 2 min prior to each electrophoretic run. A 2 min buffer rinse was used to flush out excess surfactant. The mobility was studied in 100 mM phosphate (pH 7.21) with 0 mM and 1000 mM Z1-Methyl added. The method of Williams and Vigh [35] was used as described in Section 3.2.6. In brief, a mixture of 0.2 mg/mL lysozyme and 0.5 mM benzyl alcohol

was injected into the capillary for 2 s and a low pressure push was applied for 4 min. A separation voltage of 4 kV was applied for 6 min followed by the injection of 0.5 mM benzyl alcohol for 2 s. A low pressure push was applied to move the peaks past the detector (214 nm). The effective mobility of the cation, μ^{C}_{eff}, is calculated from the equation

$$\mu^{C}_{eff} = \frac{L_T L_d (t_{N1} - t_C)}{V_{appl}(t_{N_2} + t_{inj}/2)(t_{migr} - t_{ramp-up}/2 - t_{ramp-down}/2)} \qquad (3.6)$$

where t_C, t_{N1} and t_{N2} are the times required to push the cation, first neutral marker and second neutral marker, respectively, past the detector. V_{appl} is the applied voltage; t_{inj} is the injection time; t_{migr} is the time the voltage is applied and $t_{ramp-up}$ and $t_{ramp-down}$ are the times required to linearly change the applied potential between zero and V_{appl} (10.2 s for this study). The average mobility of five replicates is corrected for viscosity effects and reported.

3.2.11 Effect of pH on EOF with Z1-Methyl

The pH of the buffer was varied to ascertain its effect on the increase in EOF caused by Z1-Methyl. The EOF was measured in 10 mM phosphate buffer at pH 2.90, 7.21 and 11.35 with 0 mM and 1000 mM Z1-Methyl added. Mesityl oxide was injected (3 s at 3.45 kPa) into a 47 cm capillary. A voltage of 20 kV was applied for a sufficient time to elute the neutral marker. The EOF was also measured in 10 mM acetate buffer pH 4.20 with 0 mM and 1000 mM Z1-Methyl added. Mesityl oxide was injected (1 s at 3.45 kPa) into a 27 cm capillary and 15 kV was applied to elute the EOF marker. The average EOF of six replicates is corrected for viscosity effects and reported.

3.3 RESULTS AND DISCUSSION

3.3.1 Monitoring Protein Adsorption

The adsorption of proteins onto the capillary wall can occur either reversibly or irreversibly. [37] Irreversible adsorption occurs when the protein adsorbs to the silica with an infinite retention factor and never reaches the detector. This type of adsorption can be measured by performing recovery studies [38] or more simply, by comparing the corrected peak areas of the protein with and without additive. Reversible adsorption occurs when the protein adsorbs onto the capillary wall but desorbs and re-enters the solution. It may be adsorbed and desorbed many times before it is detected, thus slowing its mobility in the same way that an analyte in chromatography experiences a retention time. Figure 3.3 illustrates the two forces at work as the protein moves through the capillary.

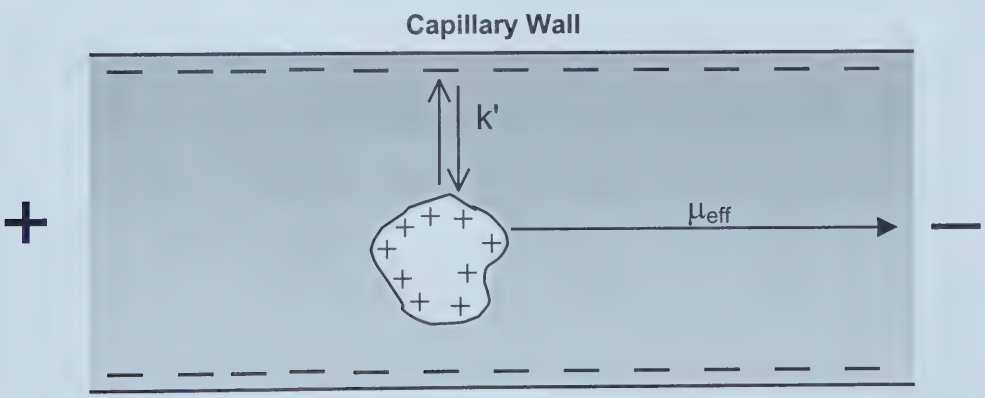


Figure 3.3 The Effect of Protein Adsorption on Mobility

This model qualitatively demonstrates that the effective mobility of the protein will decrease as the retention factor increases. On the other hand, if the protein does not adsorb to the wall, the retention factor will be zero and the protein moves to the detector

with its true effective mobility. Therefore, monitoring the effective mobility of the protein with and without additive is a reasonable indication of the extent of reversible adsorption.

3.3.2 Use of Ionic Strength to Reduce Protein Adsorption

The effective mobility of lysozyme was studied in 50 to 175 mM phosphate buffer (pH 7.21) in an uncoated capillary to investigate the effect of increasing ionic strength on protein adsorption. Experimental details are given in Section 3.2.4 and the viscosity-normalized effective mobilities are plotted in Figure 3.4. The viscosities of the buffers were measured relative to the 100 mM phosphate buffer as described in Section 3.2.3.

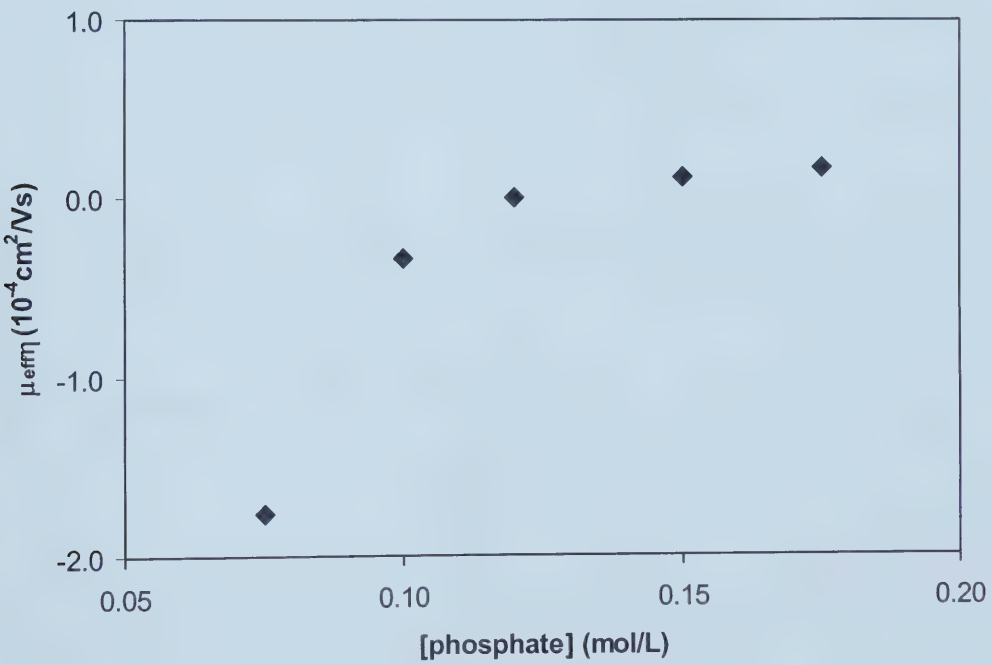


Figure 3.4 The Effect of Phosphate Concentration on the Effective Walden Product of Lysozyme. Refer to Section 3.2.4 for experimental conditions.

The use of $\mu_{eff}\eta$ (the Walden product) [39] factors out the influence of viscosity so that the effects of ionic strength can be studied. There is an increase in the effective mobility of lysozyme from negative values where it migrated after the EOF to positive values where it migrated before the EOF. The effective mobility approaches an asymptotic value at high buffer concentrations, suggesting that the retention is approaching zero. An extremely broad and tailed peak was observed from about 14 to 20 min in the 50 mM phosphate buffer. This peak was probably lysozyme, but a migration time could not be discerned since the peak was very wide and close to the baseline. The electropherograms obtained in 75 mM and 175 mM phosphate are shown in Figure 3.5.

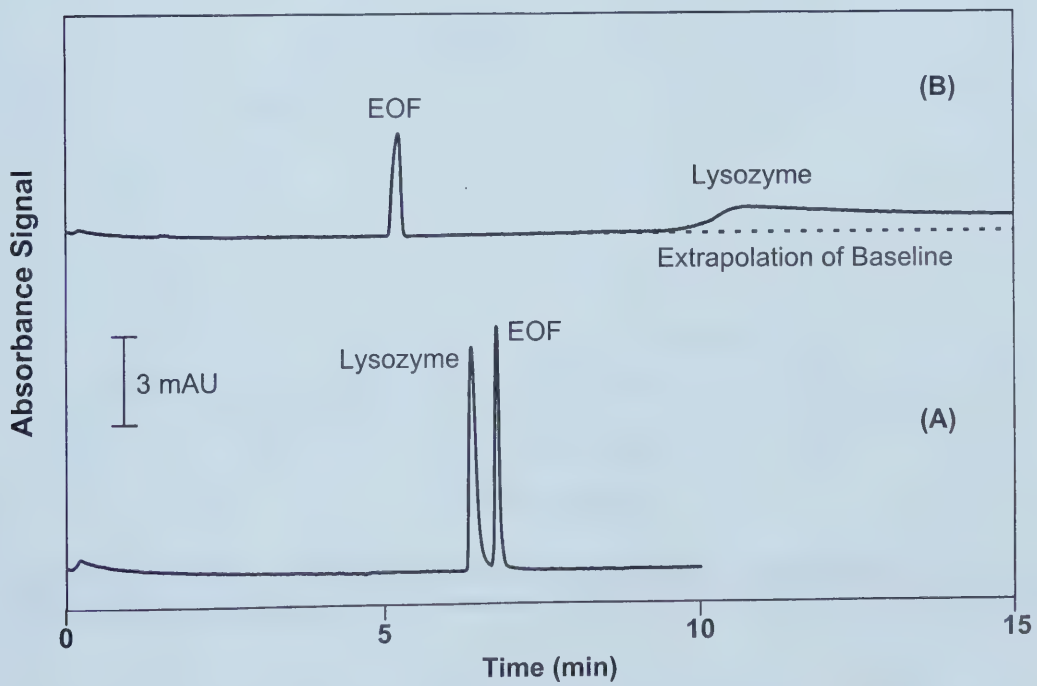


Figure 3.5 Electropherograms in (A) 75 mM phosphate buffer pH 7.21 and (B) 175 mM phosphate buffer pH 7.21. Refer to Section 3.2.4 for experimental conditions.

A migration time for the lysozyme peak could be obtained for 75 mM phosphate buffer, but peak shape is still very poor. It is much improved in the 175 mM phosphate buffer where lysozyme migrates before the EOF as expected for a positive protein. The increasing trend in mobility and improvement in peak shape suggest that ionic strength is effective in decreasing protein adsorption. However, the use of high buffer concentrations is inconvenient due to the high currents which result. A plot of current as a function of additive concentration is shown in Figure 3.6.

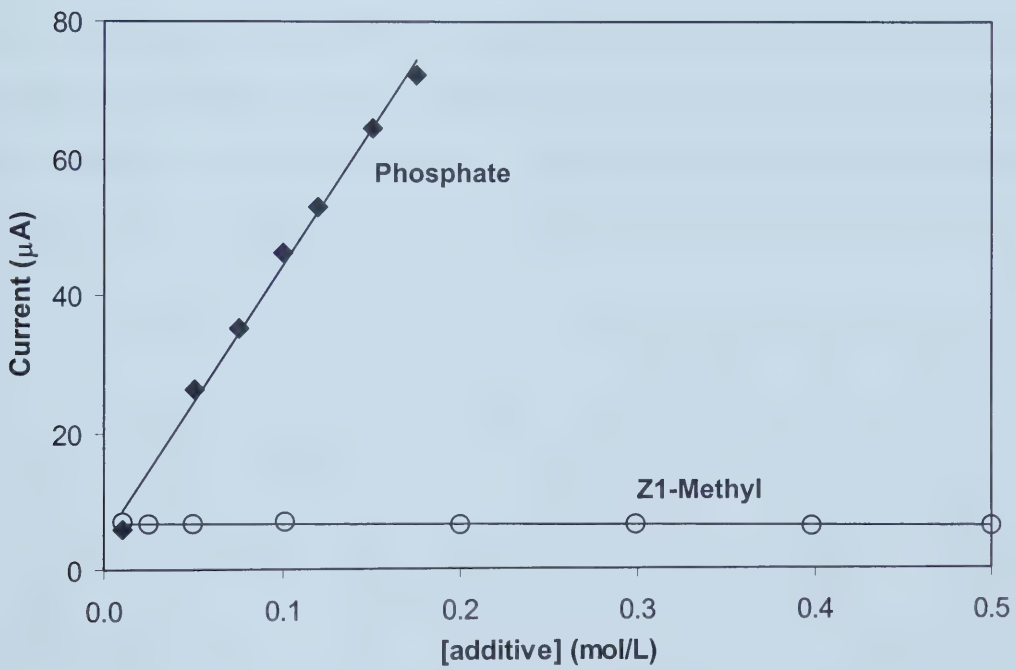


Figure 3.6 The Effect of Phosphate (◆) and Z1-Methyl (O) Concentration on Current — represents a linear regression. Refer to Section 3.2.4 for experimental conditions.

Values for current were recorded from electropherograms obtained using the conditions described in Section 3.2.4. It is obvious that current dramatically increases with phosphate concentration. To prevent Joule heating which is pronounced at high

currents, applied field strengths are limited to 185 V/cm. On the other hand, the current remains constant as the zwitterion Z1-Methyl (trimethylammoniumpropane sulfonate) is added. Therefore, the voltage limitations in high phosphate concentrations do not pertain to zwitterions. As such, significant amounts of zwitterion can be added to the buffer without increasing the current or Joule heating.

3.3.3 Use of Z1-Methyl to Reduce Protein Adsorption

Since zwitterions are reputed to be an alternative to high buffer concentrations in preventing protein adsorption, [19, 20] the utility of Z1-Methyl as a buffer additive was investigated. The effective mobility of lysozyme was studied in 100 mM phosphate buffer (pH 7.21) with 200 to 1000 mM Z1-Methyl. Experimental conditions are in Sec. 3.2.4 and the results along with those of the previous study (Sec. 3.3.2) are plotted in Figure 3.7.

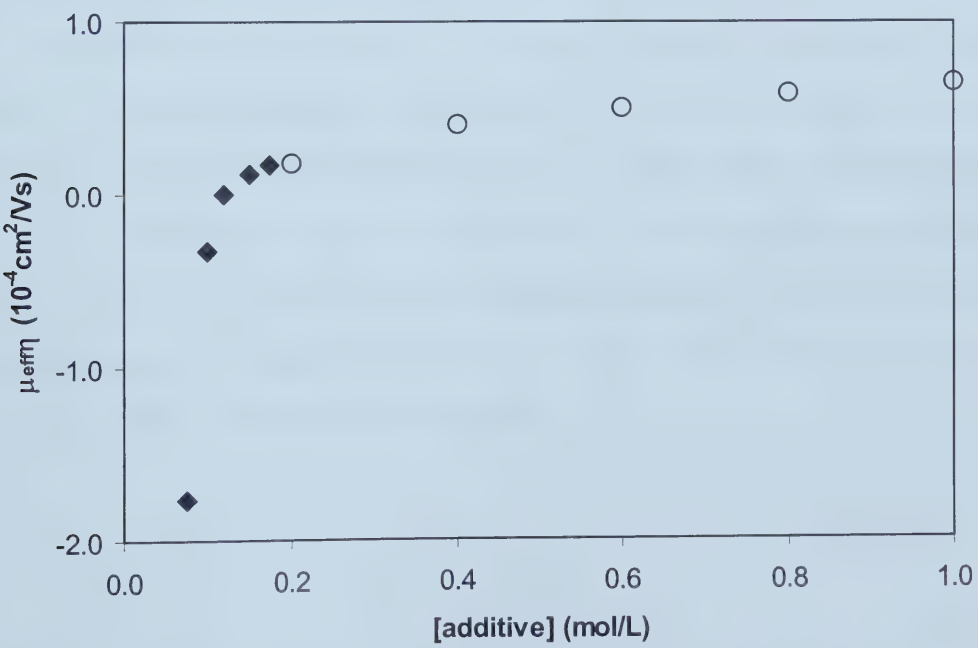


Figure 3.7 The Effect of Increasing Concentration of Phosphate (◆) and Z1-Methyl (O) on the Effective Walden Product of Lysozyme. Refer to Section 3.2.4 for experimental conditions.

The effective mobility of lysozyme continues to increase with the addition of Z1-Methyl indicating that the retention is being further reduced. The viscosity corrected mobility is leveling off at a value of $(+0.652 \pm 0.003) \times 10^{-4}$ cm^2/Vs. Thus, Z1-Methyl is beneficial in reducing protein adsorption without requiring the extremes of 175 mM buffer. Even so, the use of 100 mM phosphate buffer in addition to 1.0 M Z1-methyl seems excessive. A more normal 10 mM phosphate buffer pH 7.21 with 1.0 M Z1-Methyl was tested as described in Section 3.2.4. In this case, no lysozyme peak was observed within the electropherogram which equates to an effective mobility more negative than -5×10^{-4} cm^2/Vs. This indicates strong adsorption since lysozyme should exhibit a positive mobility. Thus, it appears that both 100 mM phosphate and 1.0 M Z1-Methyl are integral in preventing protein adsorption.

3.3.4 Protein Separations using Z1-Methyl

The electrophoretic separation of a mixture of basic and acidic proteins was obtained in 100 mM phosphate buffer at pH 7.21 with 1.0 M Z1-Methyl added. Experimental conditions are described in Section 3.2.5. Figure 3.8 shows the separation and Table 3.3 outlines the numerical analysis data. The large peak-like disturbance coincident with the EOF was observed in all buffers containing Z1-Methyl and is likely due to a refractive index change. [40] It has the same migration time as mesityl oxide and benzyl alcohol and can be used as an EOF marker.

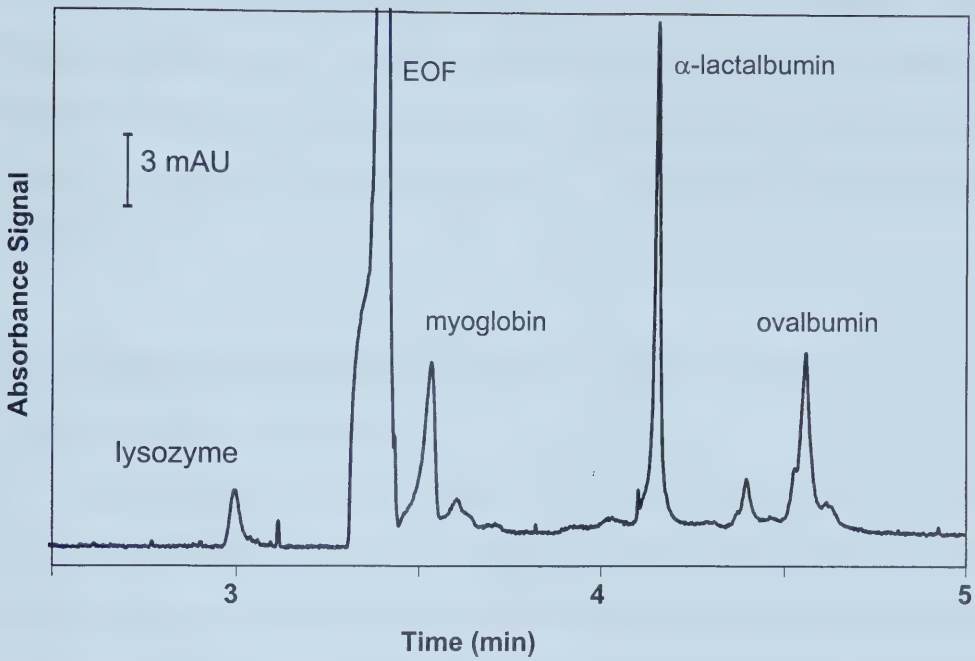


Figure 3.8 Protein Separation using 100 mM phosphate buffer pH 7.21 with 1.0 M Z1-Methyl. Refer to Section 3.2.5 for experimental conditions.

Table 3.3 Numerical Analysis Data for Protein Separation (Figure 3.8)

Analyte	Efficiency (plates/m)	Efficiency % RSD n=6	Average Migration Time (min) n=6	Migration Time % RSD n=6
Lysozyme	7×10^4	17	3.01	0.8
Myoglobin	1.0×10^5	13	3.56	0.8
α-lactalbumin	3.6×10^5	14	4.19	0.9
Ovalbumin	8×10^4	26	4.60	0.9
EOF	-	-	3.42	0.9

Lysozyme peak shape has dramatically improved over that obtained in pure phosphate buffer. Efficiencies range from 70 000 to 360 000 plates/m compared to the efficiency of lysozyme obtained in 100 mM phosphate (800 plates/m). Even though the plate count falls short of the million plates/m obtained using dynamic poly(vinyl alcohol) coatings [14]

or surfactant additives,[9] it is much improved with the addition of Z1-Methyl. While the zwitterionic additive does not eliminate protein adsorption, it appears to reduce the interaction. However, the question remains of how this is possible. In Section 3.3.5, a number of hypotheses for Z1-Methyl's ability to reduce protein adsorption will be explored.

3.3.5 Investigating the Mechanism for Reduced Protein Adsorption

3.3.5.1 Electrostatic Screening

Several plausible mechanisms may explain the reduction of protein adsorption caused by Z1-Methyl. The first possibility is that Z1-Methyl acts by providing electrostatic screening, whereby the protein is shielded from the wall charge by the ions in solution. If this were the case, the protein would be similarly shielded from the electrode to which it is attracted. A decrease in its effective mobility would result. These ionic strength effects on mobilities have been extensively studied in our group. [41] To test this hypothesis, the effective mobility of five inorganic anions were studied since these anions will not experience any retention due to adsorption. The mobility of BSA, a negatively charged protein which should not adsorb to the negative wall, was also monitored before and after the addition of 1.0 M Z1-Methyl to the 100 mM phosphate buffer. Experimental conditions are described in Section 3.2.6 and the results are presented in Table 3.4 on the following page.

Table 3.4 Effect of Z1-Methyl on the Walden Product of Anions

Analyte	Walden product: $-\mu_{eff}\eta$ (10^{-4} cm^2/Vs)		% Change in Walden Product
	0 M Z1-Methyl	1.0 M Z1-Methyl	
Bromide	6.66 ± 0.02	6.76 ± 0.02	+1.5 ± 0.3
Nitrite	6.11 ± 0.03	6.63 ± 0.02	+8.4 ± 0.5
Nitrate	5.876 ± 0.003	6.27 ± 0.05	+6.6 ± 0.9
Iodide	6.614 ± 0.001	6.26 ± 0.03	-5.3 ± 0.4
Thiocyanate	5.55 ± 0.02	5.50 ± 0.03	-0.9 ± 0.6
BSA	1.143 ± 0.002	1.354 ± 0.004	+18.5 ± 0.4

The inorganic anions do not show a consistent change in effective Walden product ($\mu_{eff}\eta$) when Z1-Methyl is added. The Walden products for bromide, nitrate and nitrite increase by 1.5, 6.6 and 8.4% respectively, while those of iodide and thiocyanate decrease by 5.3 and 0.9% respectively upon the addition of Z1-Methyl. Although the differences are significant at the 95% confidence interval, the Walden products do not exhibit an obvious decrease. In contrast, BSA exhibits a marked increase in effective mobility of 18.5% upon the addition of Z1-Methyl. This is inconsistent with the large decrease expected if electrostatic screening occurs. Further, the presence of electrostatic screening should also decrease the EOF. The EOF was monitored in 10 mM phosphate buffer at pH 7.21 as a function of Z1-Methyl concentration. Experimental conditions are given in Section 3.2.7 and results are shown in Figure 3.9 on the following page. EOF as a function of increasing phosphate concentration (data obtained in Section 3.3.2) is also shown to represent the normal effect of electrostatic screening on EOF. Increasing the concentration of phosphate buffer causes a 48% decrease in corrected EOF which is the expected trend for normal ionic strength behaviour. In contrast, the addition of 1.0 M of the zwitterion actually increases the EOF by 71% when corrected for viscosity effects! This increase is in complete contradiction to the ionic strength effect demonstrated by phosphate. Therefore, electrostatic screening is not an

acceptable explanation for the reduction in protein adsorption. An explanation for the increase in effective mobility and electroosmotic flow will be given in Sections 3.3.8 to 3.3.11.

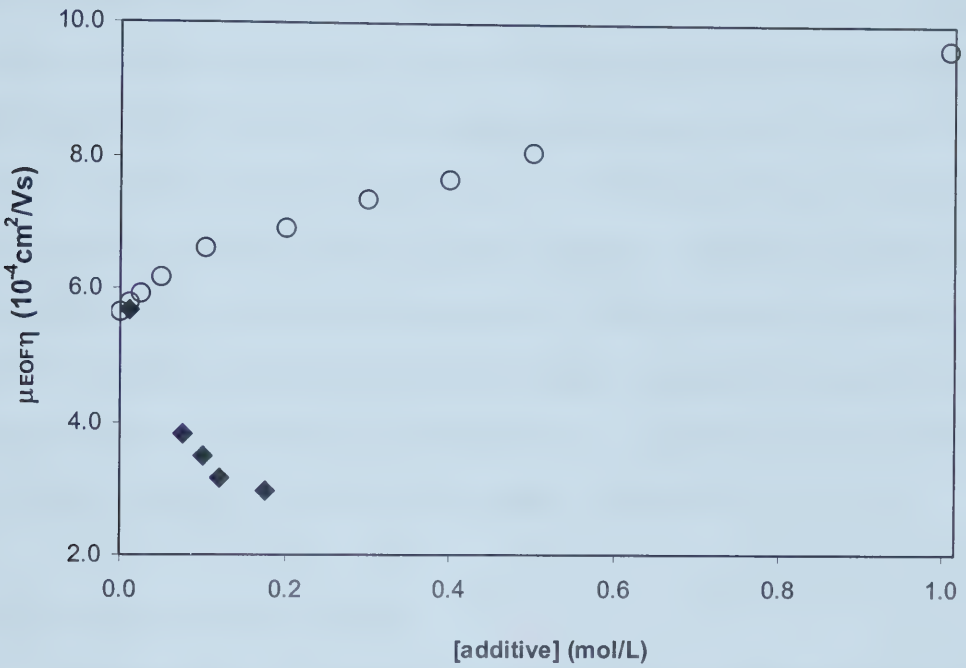


Figure 3.9 Effect of Z1-Methyl (O) and Phosphate (◆) Concentration on EOF. Refer to Section 3.2.4 for experimental conditions.

3.3.5.2 Reduction in Wall Charge

The Coulomb attractive force between the positively charged moieties of the protein and the negatively charged wall play a primary role in protein adsorption. This force is given by [42]

$$F = \frac{Q_P Q_W}{4\pi\varepsilon_0\varepsilon} \qquad (3.7)$$

where Q_p and Q_w are the protein charge and wall charge respectively, ε_0 is the dielectric constant in vacuum and ε is the dielectric constant of the buffer. A reduction in the wall charge will reduce the Coulombic force and as a result, reduce the amount of protein adsorbing onto the wall. Since the EOF is a consequence of the negatively charged silanol groups on the wall (see Sec. 1.3.2), a reduction of wall charge will simultaneously decrease the EOF. Figure 3.9 clearly displays an increase in EOF. In fact, a 71% increase is observed at high concentrations of Z1-Methyl. Similar increases in electroosmotic flow with the use of Z1-Methyl have been reported in the literature. [40] This implies that the wall charge, if altered at all, must be increased to account for the larger EOF. Since this would promote further protein adsorption, a reduction in wall charge is not a valid explanation for Z1-Methyl efficacy. An explanation for the increase in EOF upon addition of Z1-Methyl will be presented in Sections 3.3.8 and 3.3.9.

3.3.5.3 Reduction in Protein Charge

Alternatively, eqn 3.7 shows that a decrease in Coulombic attraction between the wall and the protein could be effected by a reduction in protein charge (Q_P). An increase in the pH of the solution will cause the protein to possess a more negative charge. However, measurement of the buffer pH before and after the addition of 1.0 M Z1-Methyl verifies that Z1-Methyl does not alter the pH of the solution. Therefore, the only means by which Z1-Methyl could alter the protein charge is to lower its isoelectric point. This could be accomplished by shifting the pK_a of the amino acid groups which make up the protein. Since proteins are composed of a multitude of amino acids, measurement of the pI is difficult. Therefore, two simple model compounds, methylamine and acetic acid, were chosen to represent the amine and carboxylic acid

moieties of the protein. A potentiometric titration was performed on solutions of methylamine and acetic acid without Z1-Methyl added to obtain the true pK_a and then repeated for the analytes when 0.5 M Z1-Methyl was added. Experimental details are supplied in Section 3.2.8 and results are shown in Table 3.5.

Table 3.5 Effect of Z1-Methyl on pK_a

Conditions	pK_a of Methylamine	pK_a of Acetic Acid
Literature value in water [29]	10.63	4.76
Experimental value in water	10.59	4.72
Experimental value in 0.5 M Z1-Methyl	10.55	4.76

The experimental pK_a values in water are 10.59 and 4.72 for methylamine and acetic acid respectively. These values agree quite well with the reported literature values of 10.63 and 4.76. [29] The pK_a of methylamine exhibits a slight decrease upon the addition of Z1-Methyl, whereas the pK_a of acetic acid shows a slight increase. Neither of these pK_a changes is large enough to cause a significant variation in dissociation or charge. Therefore, it is inferred that Z1-Methyl does not reduce the protein charge by altering the dissociation constants of its amino acids.

Furthermore, if Z1-Methyl had affected the pK_a of either the amine or the carboxylate functionality, it should do so consistently. That is, if the pK_a of the amine were increased, this should increase the charge of cationic proteins (i.e., proteins below their pI) and increase their mobility. This is the behaviour noted for lysozyme in Figure 3.7. In the same manner, the pK_a effect would have to lower the charge of anionic proteins (i.e., proteins above their pI), and thus lower their mobility. However, in Table

3.4, the viscosity corrected mobility of the anionic protein BSA was observed to increase 18.5% upon the addition of 1.0 M Z1-Methyl.

Thus, no significant pK_a shifts were observed for model amines and carboxylic acids upon the addition of Z1-Methyl. Moreover, the effect of Z1-Methyl on the mobility of cationic and anionic proteins is not consistent with any systematic pK_a shift. In conclusion, the hypothesis that Z1-Methyl operates by altering the protein charge can be eliminated.

3.3.5.4 Competition for Cation Exchange Sites

Researchers in the literature have speculated that zwitterions decrease adsorption by competing with the protein for ion association sites on the negative silanol groups. [23, 24, 26] Such a competition would decrease the adsorption of positive proteins which are retained by these sites. This would cause an increase in effective mobility as observed for lysozyme in Figure 3.7. However, it would not explain why Z1-Methyl increases the effective mobility of negative analytes such as BSA which are not retained by a cation exchange site.

Also, if Z1-Methyl somehow did compete for the ion exchange sites, it would presumably replace the cations in the double layer that are responsible for the EOF. It would seem reasonable that such a displacement by a zwitterion possessing no mobility would result in a decrease in EOF. Yet, as shown in Figure 3.9 and discussed in Section 3.3.5.2, the EOF actually increases upon addition of Z1-Methyl!

Furthermore, if Z1-Methyl were to compete with cations for silanols on the capillary wall, the ion exchange capability of the cation would impact the resulting EOF change. To investigate this possibility, the EOF was monitored while using different

buffer cations to generate the EOF. Electroosmotic flow was measured in 10 mM phosphate buffer pH 7.21 with lithium, sodium and potassium counter-ions before and after the addition of 0.25 M Z1-Methyl. Experimental conditions are given in Section 3.2.9 and results are outlined in Table 3.6.

Table 3.6 The Effect of Cation on the EOF Increase with Z1-Methyl

Buffer	EOF (10^{-4} cm^2/Vs) with 0 M Z1-Methyl	EOF (10^{-4} cm^2/Vs) with 0.25 M Z1-Methyl corrected for viscosity	% EOF Increase
10 mM lithium phosphate pH 7.21	5.48 ± 0.06	6.83 ± 0.04	25 ± 2
10 mM sodium phosphate pH 7.21	5.67 ± 0.04	7.13 ± 0.02	26 ± 1
10 mM potassium phosphate pH 7.21	5.26 ± 0.03	6.60 ± 0.01	25 ± 1

A 25 to 26% increase in EOF with the addition of Z1-Methyl is observed for all cations. One would expect a larger increase in EOF for lithium (a weak ion exchange cation) compared to potassium (a stronger ion exchange cation), [43] if a competition type mechanism was at work. Instead, the increase in EOF remains constant as the cation is varied. Thus, the possibility of Z1-Methyl decreasing adsorption by competing for silanol ion exchange sites has been disproved.

3.3.6 A Second Look at the Reduction of Protein Adsorption

Every hypothesis to explain how Z1-Methyl reduces protein adsorption has been refuted. At this time, the tenet on which we base the efficacy of Z1-Methyl must be scrutinized. Is the observation of a lysozyme peak with an increased effective mobility sufficient evidence to support a reduction in *k'* and thus adsorption?

It would be informative to have a value for the effective mobility of lysozyme where it is certain that wall adsorption has been eliminated. Therefore, the effective mobility of lysozyme was determined in a capillary coated with DDAB (Fig. 3.1C). This is a double chained positive surfactant which has been shown to prevent the adsorption of positive proteins including lysozyme. [44] DDAB forms a bilayer coating on the wall which remains stable even when it is removed from the running buffer. This is beneficial because there is no danger of free surfactant in solution affecting the mobility of the protein. Experimental conditions are described in Section 3.2.10.

The effective mobility of lysozyme in the absence of adsorption effects is $(0.499 \pm 0.002) \times 10^{-4}$ cm^2/Vs in 100 mM phosphate buffer pH 7.21. In comparison, the viscosity corrected effective mobility of lysozyme in 100 mM phosphate pH 7.21 with 1.0 M Z1-Methyl added is $(0.652 \pm 0.003) \times 10^{-4}$ cm^2/Vs. This value is higher than the reference mobility! Even if DDAB were to affect the reference mobility, it would be biased higher since association with the positively charged surfactant will increase the charge-to-size ratio of the protein. The mobility in Z1-Methyl is actually higher than the mobility in DDAB. Since this does not make fundamental sense, there must be a flaw in the assumption that an increase in effective mobility is caused by a decrease in protein adsorption.

3.3.7 Calculation of Retention Factor

Comparison of mobility data is not the most reliable method to evaluate the effectiveness of Z1-Methyl in preventing protein adsorption. Mobility is affected by a variety of solution properties which are difficult to control. A method which compares the retention factor of the protein before and after the addition of Z1-Methyl would

provide a more infallible approach. The following equation describing retention in chromatography will be adapted to protein adsorption in capillary electrophoresis. [45]

$$k' = \frac{t_r - t_0}{t_0} \qquad (3.8)$$

where t_r is the retention time of the protein which is being adsorbed to the wall and t_0 is the elution time of the same protein when it is not adsorbed. It is trivial to obtain a migration time for lysozyme in Z1-Methyl to use as the retained time since it is obvious the protein is adhering to the wall. However, a migration time in the same capillary and buffer where it is certain lysozyme does not adsorb is more complex and accomplished as follows. Table 3.7 outlines the data used throughout these calculations and the resultant retention factors.

Table 3.7 Calculation of Retention Factor of Lysozyme

	100 mM phosphate pH 7.21		100 mM phosphate pH 7.21 1.0 M Z1-Methyl	
Calculated Parameter	DDAB coated capillary	Uncoated Capillary	DDAB coated capillary	Uncoated capillary
μ_{eff} (10^{-4} cm^2/Vs)	0.54 ± 0.02		0.574 ± 0.009	
μ_{EOF} (10^{-4} cm^2/Vs)		3.075 ± 0.004		3.338 ± 0.006
μ_{app} (10^{-4} cm^2/Vs)		3.62 ± 0.02		3.91 ± 0.01
t_o (min)		3.28 ± 0.02		3.034 ± 0.008
t_r (min)		3.860 ± 0.006		3.555 ± 0.003
k' (unitless)		0.18 ± 0.04		0.17 ± 0.02

The effective mobility of lysoyzme from the DDAB coated capillary with Z1-Methyl in the buffer affords the mobility in the absence of protein adsorption but in the same buffer system. To relate this to the uncoated capillary, the EOF in the bare capillary will be used in eqn 3.3 to calculate the apparent mobility in this capillary. Finally, the

substitution of the capillary length and voltage of the uncoated capillary into eqn 3.2 will provide an unretained migration time for lysozyme. Use of the same buffer system eliminates any variance in solution properties such as viscosity. At last, the retention factor for lysozyme in 100 mM phosphate (pH 7.21) with 1.0 M Z1-Methyl was obtained using eqn 3.8. The entire process was repeated to obtain a retention factor in the 100 mM phosphate buffer system without Z1-Methyl added. Errors in Table 3.7 are the standard deviation of the replicates performed and are propagated throughout the calculations.

The non-zero retention factors indicate that the problem of protein adsorption remains. In fact, retention factors as low as 0.05 can reduce protein efficiencies by a factor of 20. [19, 46] Moreover, the retention factors with and without Z1-Methyl agree well within the 95% confidence level. The addition of the zwitterion does not reduce wall adsorption effects beyond what has already been accomplished by the large phosphate concentration.

3.3.8 Explaining the Effect of Z1-Methyl

The preceding discussion indicates that Z1-Methyl is ineffective in decreasing protein-wall interactions. As such, the increase in effective mobility of lysozyme cannot be attributed to a decrease in protein adsorption. A look at the theory describing mobility in capillary electrophoresis may provide insight into the increase in effective mobility and EOF upon addition of Z1-Methyl. The Hückel equation for the electrophoretic mobility of a small particle is given by [47]

$$\mu = \frac{2}{3}\frac{\zeta\varepsilon}{\eta} \qquad (3.9)$$

where ζ is the zeta potential of the particle, ε is the dielectric constant of the solution, and η is the solution viscosity. Similarly, the electroosmotic flow in the capillary is given by

$$\mu_{EOF} = -\frac{\zeta\varepsilon}{\eta} \qquad (3.10)$$

where ζ is the zeta potential of the capillary wall and ε and η are the same as above. These equations indicate that a decrease in viscosity would cause an increase in both mobility and EOF. However, this explanation is not feasible since Z1-Methyl increases viscosity and all EOF and electrophoretic mobilities above have been corrected for these viscosity changes.

Alternatively, an increase in dielectric constant would result in an increase in both mobility and EOF. In fact, increases in dielectric constant with increasing concentration of zwitterion have been reported in the literature. [48-51] The abnormally high dielectric constants of zwitterions is what initially gave evidence that these ampholytes exist in the zwitterion form. [49] The dielectric increment, δ, describes the change in dielectric constant, ε, per change in concentration, C, of additive

$$\delta = \frac{\Delta\varepsilon}{\Delta C} \qquad (3.11)$$

Accordingly, the dielectric constant of a solution, ε, is given by [49]

$$\varepsilon = \varepsilon_W + \delta C \qquad (3.12)$$

where ε_W is the dielectric constant of pure water at 25 ^{0}C (78.48 [52]), δ is the dielectric increment and C is the concentration of additive. The dielectric increment for zwitterions is positive resulting in higher dielectric constants, whereas the increment of organic solvents such as methanol is negative causing a decrease in dielectric constant. [48] Figure 3.10 illustrates how the dielectric constant varies with concentration of α-aminobutanoic acid. [49] Equation 3.12 exhibits excellent linearity up to 2.0 mol/L of zwitterion. Since the data presented in this work is well within this concentration range, the use of eqn 3.12 is valid.

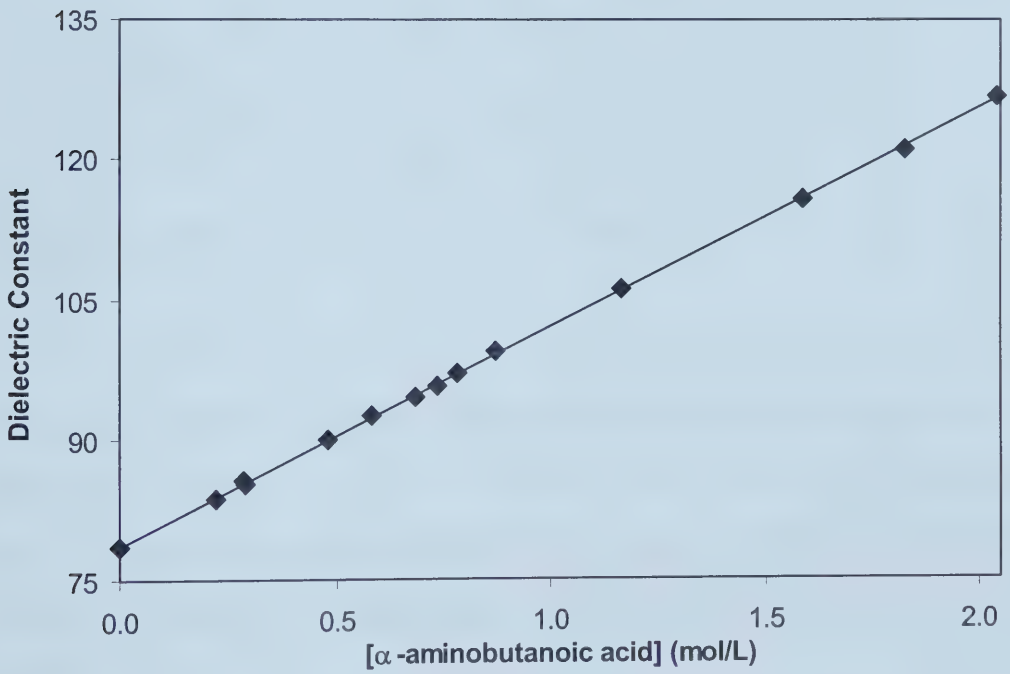


Figure 3.10 Effect of α-aminobutanoic Acid Concentration on Dielectric Constant. ◆ represents experimental data and — represents linear regression by equation: ε = 78.54 + 23.53*C. Data and equation from reference 49, Table 1.

Kirchnerova et al. report the dielectric increment of 3-aminopropane sulfonate to be 56.8. [48] This compound differs from Z1-Methyl only by the lack of a quaternary methyl-substituted amine. Wyman states that the dielectric increment of the hydrogen-substituted and the methyl-substituted amine are nearly identical. [50] Table 3.8 shows the dielectric increments of some amino acids and their trimethyl-substituted (betaine) counterparts.

Table 3.8 Dielectric Increments of Amino Acids [48]

Compound	δ	δ of the betaine	% Difference
Glycine	24	21.8	9 %
4-aminobutanoic acid	53	44.4	16 %
5-aminovaleric acid	63	60	5 %
6-aminohexanoic acid	82	73.5	10 %
7-aminoheptanoic acid	93	89.2	4 %
3-aminopropane-sulfonate	56.8	unknown	
3-triethylammonium-propane sulfonate		58.6	

The average decrease in dielectric increment upon going to the betaine form of the amino acid is 9 (± 5) %. Using this average decrease and the dielectric increment for 3-aminopropanesulfonate, the increment of its betaine form (Z1-Methyl) is estimated to be 52 ± 3. Galin reports the dielectric increment for triethylammoniumpropane sulfonate to be 58.6. [51] Therefore, an estimate of 52 ± 3 for the trimethyl-substituted zwitterion is quite reasonable.

Based on the above, the addition of 1.0 M Z1-Methyl should cause a 66 ± 4% increase in the dielectric constant from 78.48 in pure water to 130 ± 3 in the zwitterionic solution.

3.3.9 Comparison of the Dielectric Constant Increase with EOF Increase

Dielectric constant has previously been used to explain changes in the EOF resulting from the addition of organic solvents such as methanol to CE buffers. [53, 54] The dielectric constant as a function of Z1-Methyl concentration was calculated from eqn 3.12. Figure 3.11 plots this relationship along with the increase in EOF observed in Section 3.3.5.1.

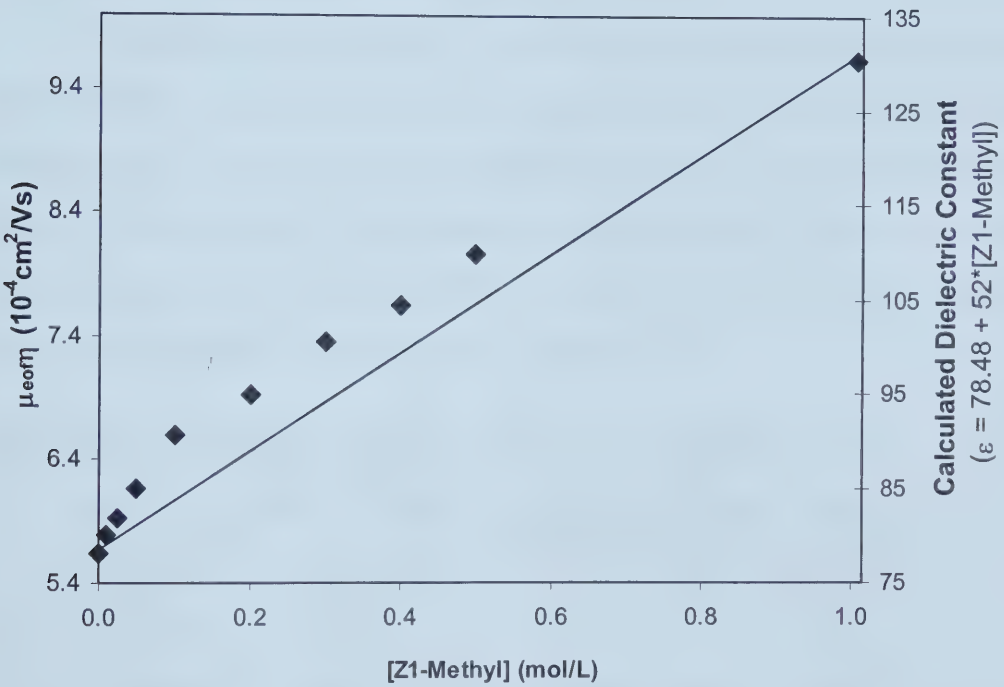


Figure 3.11 Effect of Z1-Methyl on EOF (◆) and Dielectric Constant (—) Refer to Section 3.2.7 for experimental conditions and Section 3.3.8 for dielectric constant calculation.

The experimental EOF displays excellent agreement with the predicted dielectric constant. The overall 71 ± 1% increase in EOF correlates very well with the increase in dielectric constant predicted for the addition of 1.0 M Z1-Methyl. Increases in

electroosmotic flow upon the addition of Z1-Methyl have also been observed by other researchers. [23, 25] As with Figure 3.11, Buchberger and Winna observed nonlinearity in the relationship between EOF and concentration of Z1-Methyl. Overall, the theory that increases in EOF are due to an increase in dielectric constant is very well supported by the experimental data.

3.3.10 Effect of pH on the EOF Increase with Z1-Methyl

If the EOF changes are due to a change in the dielectric constant of the buffer, pH should have no effect on the relative increase in the EOF. To test this assumption, the effect of pH on the EOF increase obtained with the use of Z1-Methyl was studied. The EOF was determined in 10 mM phosphate buffer at pH 2.90, 7.21 and 11.35 before and after the addition of 1.0 M Z1-Methyl. Experimental details are given in Section 3.2.11 and the EOF data are summarized in Table 3.9.

Table 3.9 The Effect of pH on the EOF Increase with Z1-Methyl

Buffer	EOF (10^{-4} cm^2/Vs) with 0 M Z1-Methyl	EOF (10^{-4} cm^2/Vs) with 1.0 M Z1-Methyl corrected for viscosity	% increase in EOF
10 mM acetate pH 4.20	3.28 ± 0.05	5.28 ± 0.06	61 ± 3
10 mM phosphate pH 7.21	5.67 ± 0.04	9.70 ± 0.08	71 ± 1
10 mM phosphate pH 11.35	6.96 ± 0.02	10.80 ± 0.02	55 ± 1

Unfortunately, the pH 2.90 buffer resulted in very low EOF which was irreproducible from capillary to capillary even in the absence of additive. Thus, the data is not presented. Irreproducible EOF at low pH has been observed previously [55] and Coufal et al. report that phosphate buffer yields the poorest reproducibility of all buffers. [56] To obtain a

measure of the effect of Z1-Methyl at low pH, 10 mM acetate buffer at pH 4.20 was used. Over the range of pH and buffers studied, the increase in EOF upon addition of 1.0 M Z1-Methyl varies from 55 to 71%, as shown in Table 3.9. No systematic trend in the EOF increase with pH is observed. Furthermore, the average increase is 62% which agrees very well with the estimated 66 ± 4% increase in dielectric constant.

3.3.11 Discussion of the Increase in Effective Mobility

The effective mobility of proteins exhibit a definite increase with the addition of Z1-Methyl. A 31 ± 1% increase in mobility was observed for lysozyme, a positive protein, upon the addition of 1.0 M Z1-Methyl. Bovine serum albumin, a negative protein, exhibited an 18 ± 1% increase in effective mobility with the addition of 1.0 M Z1-Methyl. Although the two proteins differ in the amount their mobilities increase, they do follow the same general increasing trend as the EOF and dielectric constant. Therefore, the increase in mobility is attributed to the increase in dielectric constant with the addition of zwitterion.

The effect of Z1-Methyl on the mobilities of inorganic anions appears to be puzzling at first glance. Table 3.4 shows that some anions experience an increase in mobility upon the addition of Z1-Methyl, whereas others exhibit a decrease. Equation 3.9 predicts a proportional increase in mobility with an increase in dielectric constant. The anomalous behaviour displayed by the inorganic anions may be caused by electrostatic CE interactions as outlined in Chapter 2. Unlike the zwitterions used in Chapter 2, Z1-Methyl is not a surfactant and will not form micelles. Nevertheless, Kaneta et al. [57] have shown that inorganic anions are retained by monomers, albeit to a lesser extent than micelles. In addition, Figure 2.6 on page 51 shows that iodide and

thiocyanate are retained by Zwittergent 3-08 which exists as monomers at the concentrations used. The use of Z1-Methyl is more complicated since there are two forces at work. Z1-Methyl will retard the mobility of inorganic anions *via* electrostatic interactions. At the same time, the increase in dielectric constant will cause an increase in anion mobility. Ions such as bromide, nitrite and nitrate are lower in the Hofmeister series and will primarily experience dielectric influences causing an increase in mobility. However, ions such as iodide and thiocyanate which are more chaotropic are dominated by electrostatic CE interactions and exhibit a decrease in effective mobility. The protein, BSA, is composed of amines and carboxylic acids which are low in the Hofmeister series. [58] Thus, the mobility of BSA is prevalently influenced by increases in dielectric constant, explaining the large mobility increase observed.

3.3.12 The Use of Z1-Methyl and Other Zwitterions in the Literature

Since the proposed reduction in protein adsorption caused by Z1-Methyl has been negated, an explanation of effects observed in the literature is necessary. The efficiency of ricin, a glycoprotein, was 75 000 plates/m when Z1-Methyl was used to reduce protein adsorption. [24] These efficiencies are still many times less than the million plates/m predicted by theory and obtained in polymer or surfactant coated capillaries. [9, 14] Furthermore, the sample concentration used was 0.5 mg/mL which is considerably higher than the typical ~0.1 mg/mL which provides more than adequate signal-to-noise when using capillaries with good wall coatings. [8] These circumstances indicate that protein adsorption had not been eliminated by the addition of Z1-Methyl.

Guzman and coworkers [23] used Z1-Methyl to prevent protein adsorption in the separation of monoclonal antibodies. Improvement in peak area and the relative

standard deviation (RSD) of peak area were used as measures of additive performance. Z1-Methyl showed an overall *decrease* in peak area from that of a bare capillary without additive and only a slight increase in area reproducibility. If protein adsorption was eliminated, an obvious *increase* in peak area should have been observed since less protein is lost to the wall.

Buchberger and Winna [25] used Z1-Methyl as an additive to reduce adsorption in the determination of fatty acids. However, separations were done at an increased temperature in a buffer that also contained 70 % ethyleneglycolmonomethylether to decrease peak tailing by adsorption. This leads one to question the utility of Z1-Methyl in preventing adsorption when such a complicated buffer system was necessary. The use of Z1-Methyl produces a 50% increase in EOF from the suppressed value caused by the organic solvent. This increase in EOF could account for the increased efficiency according to eqn 3.1.

Fang and Sun [21] attained a 20-fold increase in the efficiency of lysozyme with the addition of 1.0 M Z1-Methyl to the buffer. This improvement may also be attributed to the increase in EOF with the use of Z1-Methyl. Furthermore, the same authors demonstrated that 2.0 M trimethylammoniumbutane sulfonate produced a 200-fold increase in efficiency. The dielectric increment of triethylammoniumbutane sulfonate, a structurally similar compound, is 75.4. [51] This would produce a 202% increase in dielectric constant and consequently, EOF, which corresponds very well with the increase in efficiency.

Next, the analysis of egg white proteins by Waters required a 10 s injection of 20-fold diluted egg white and the use of both 1.0 M Z1-Methyl and 350 mM phosphate buffer! [22] The usefulness of Z1-Methyl in such a high ionic strength buffer system is

also disputable since protein adsorption would already be significantly reduced by ionic screening from the 350 mM buffer.

The utility of Z1-Methyl in the separation of monoclonal proteins by Miura et al. [27] is also questionable. A pH of 10 was employed which exceeded the pI of the proteins, resulting in full deprotonation. The use of high pH with no additive has previously been shown to effectively reduce protein adsorption. [3] In addition, the use of pH 4.0 produced no protein peaks indicating that pH played the major role in preventing adsorption.

Finally, the use of glycine and betaine (Fig. 3.1B) by Jorgenson [19] provided only minimal increases in efficiency. The use of 2 M glycine did not produce any useful peaks. The addition of 2 M betaine to a buffer containing 0.1 M potassium sulfate increased the efficiency of lysozyme from 28,700 plates to 128,000 plates. This increase may be attributed to the increased viscosity (estimated at 60% higher in 2 M betaine from the current data provided). Viscosity increases lower the diffusion term in eqn 3.1, thereby increasing the number of plates. This is also supported by the fact that 2 M betaine alone yielded only 3700 plates for the protein.

In all of the cases discussed above, the improvement in efficiency and peak shape is not substantial and the issue is confused by complex buffer systems or high sample concentrations. The efficacy of Z1-Methyl in preventing protein adsorption is, at best, minimal.

3.4 CONCLUDING REMARKS

The addition of Z1-Methyl to the buffer causes increases in the electroosmotic flow as well as the effective mobility of the analytes. These phenomena have been explained by the linear increase in dielectric constant with concentration of zwitterion added to the buffer. Mechanisms to explain the proposed efficacy of Z1-Methyl in preventing protein adsorption have been demonstrated to be inadequate. The apparent reduction in protein adsorption attributed to Z1-Methyl has also been rationalized by the increase in dielectric constant. In essence, zwitterionic additives such as Z1-Methyl offer no significant benefits with regard to protein adsorption. However, the enhanced electroosmotic flow and electrophoretic mobility associated with the addition of zwitterion to the buffer may provide unique opportunities in CE.

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CHAPTER 4: CONCLUSIONS

The application of zwitterions in a variety of capillary electrophoresis separations has been demonstrated. Zwitterions possess both positive and negative functional groups giving an overall charge, and thus conductivity, of zero. As a result, high concentrations of these species can be added to the background electrolyte without inducing Joule heating.

Chapter 2 described a new technique to alter the selectivity of inorganic anion separations in CE. The addition of the zwitterionic surfactant, 3-(N,N-dimethyl-dodecylammonio)propane sulfonate (DDAPS) to the separation buffer altered the migration order of inorganic anions *via* electrostatic ion chromatography effects. At low DDAPS concentrations, minor selectivity changes were observed similar to those obtained with a cationic surfactant. The advantage of DDAPS over cationic surfactants is the ability to use high concentrations without increasing current or Joule heating, or interfering with indirect detection. Moreover, high concentrations of DDAPS caused dramatic selectivity changes. The resultant migration order was the same as that observed in electrostatic ion chromatography.

The mechanism of electrostatic ion chromatography was used to explain the retention of anions by DDAPS. In this mechanism, buffer ions associate with oppositely charged groups on the zwitterionic surfactant. This establishes a charged layer or Donnan membrane which analyte anions must overcome in order to interact with the quaternary ammonium group of the surfactant. Retention of the anion is governed by chaotropic interaction with the positive group according to the Hofmeister series. This

explains why more polarizable and chaotropic ions such as iodide and thiocyanate are most significantly retained.

Adjustment of the DDAPS concentration alters the migration order of the analytes, providing the analyst with a powerful separation tool. To demonstrate its flexibility, the method was applied to the separation and quantification of inorganic anions in artificial seawater. Seawater poses a formidable challenge to CE separations since chloride usually co-migrates with and obscures the analyte peaks of interest. This problem was solved by using an appropriate concentration of DDAPS to remove the peaks from the area of interference. Detection limits of nitrate, nitrite, bromide and iodide in 1:5 diluted artificial seawater were 11, 5, 7 and 11 μM, respectively.

Chapter 3 investigated the reduction in protein adsorption provided by the zwitterion Z1-Methyl. A variety of mechanisms were examined to explain the perceived reduction in protein adsorption. None were successful. Therefore, the retention factor was determined for the adsorption of lysozyme to the silica wall in the absence and presence of Z1-Methyl. The retention factor was the same for both systems indicating Z1-Methyl was ineffective in preventing protein adsorption.

The addition of a high concentration of zwitterion to a solution causes an increase in the dielectric constant. In the literature, an increase in the effective mobility and efficiency of the protein have been used as indicators of reduced protein-wall interaction. The dielectric constant increase explains the increase in effective mobility according to the Hückel equation. Furthermore, proteins which migrate faster spend less time in the column and have less of an opportunity for adsorption. This explains the increased efficiency observed with the addition of Z1-Methyl.

An intriguing phenomenon caused by the addition of Z1-Methyl was an increase in EOF by up to 40 %. This behaviour can be explained by the von Smoluchowski equation since an increase in dielectric constant will increase the electroosmotic flow. The dramatic EOF increase presents unique opportunities for capillary electrochromatography (CEC) where the solution flow is EOF driven. Furthermore, extending the chain length which separates the two charged groups in a zwitterion would enhance the increase in dielectric constant. [1] Exploring other zwitterions for their dielectric properties and ability to increase EOF would be an interesting future project. Finally, EOF can be monotonically increased with the addition of zwitterion to the buffer. Thus, the researcher is able to fine-tune the magnitude of the EOF without the cost of increased conductivity. This is a valuable tool in separations where mobility counterbalance is required for resolution of analytes. [2-4]

In summary, zwitterions can be used in many capillary electrophoresis applications. High concentrations can be employed without contributing to band broadening caused by Joule heating. This thesis has demonstrated the use of zwitterions in selectivity alteration and explained the effects of zwitterions on protein adsorption and EOF.

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